IAW

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re U.S. Patent Application of

Applicant: Cindy ORSER et al.

Application No.: 10/728,246

Filing Date: 4 December 2003

Confirmation No.: 8108

Title: DETECTION OF CONFORMATIONALLY ALTERED PROTEINS AND PRIONS

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

PETITION TO MAKE SPECIAL

Sir:

Applicants request that this application be accorded Special Status on the basis of the age of inventor Eugene A. DAVIDSON.

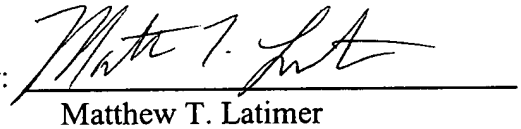
As stated on the attached Statement of inventor DAVIDSON, he is currently 65 years of age or older. In accordance with 37 C.F.R. § 1.102(c), no fee should be required for entry and consideration of this Petition.

The currently pending claims, which have not been examined, are all directed to a single invention.

A pre-examination search has been performed on the claims, as now pending. The results and search statements for the search are attached as part of an Appendix to this document, as are copies of the references deemed most relevant and a discussion of the references.

Respectfully submitted,
Cindy S. ORSER et al.

Date: 29 March 2006

By: 
Matthew T. Latimer
Reg. No. 44,204

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STATEMENT OF INVENTOR DAVIDSON REGARDING SPECIAL STATUS

Sir:

In connection with the Petition to Make Special filed herewith, I state that:

1. I am an inventor of the above-referenced patent application;

2. I am currently 75 years of age, having been born on May 27, 1930.

3. I believe that this Statement satisfies the requirements for a Statement of an Inventor to support Special Status of this application, without the need for payment of a fee.

Respectfully submitted,
Eugene A. DAVIDSON

Date: 3/31/06

Eugene A. Davidson

APPENDIX - PETITION TO MAKE SPECIAL
10/728,246

I. Fields of Search

A. USPTO granted patents database:

1. - Keyword search (terms varied to create numerous statements, using ACLM and SPEC fields) for general concept:
prion$, (detect$ or identif$ or determ$), (assay or method)
- Keyword search for particular concepts specifically recited in claims:
search strategies attached as "Keyword Search Terms"

2. Classes/subclasses:
257/784; 257/741
422
424/520; 424/529; 424/530; 424/531
435/2; 435/4; 435/5; 435/7.1
436; 436/536; 436/543
455/561
514/2; 514/12; 514/13; 514/14; 514/15
530/300; 530/324; 530/326; 530/327; 530/350; 530/380

B. USPTO patent application publications database

1. - Keyword search for general concept:
prion$, (detect$ or identif$ or determ$), (assay or method)
- Keyword search for particular concepts specifically recited in claims:
search strategies attached as "Keyword Search Terms"

C. EPO and WIPO Websites

1. - Keyword search for general concept:
prion$, (detect$ or identif$ or determ$), (assay or method)
- Keyword search for general concepts specifically recited in title or abstract:
search strategies attached as "Keyword Search Terms"

D. Medline and STN searches:
Keywords as detailed above, in numerous different permutations

E. Dgene and nlm GenBank searches:

1. All SEQ ID Nos of application
2. Re-search with VVAGAAAAGAMHKM

Keyword Search Terms

PGPs from USPTO website:

pyrene AND excimer AND (conformation OR conformational): 34 applications
aclm/fluorescence AND aclm/(protein OR prion) AND (conformation OR conformational) : 519 applications
aclm/fluorescence AND aclm/prion AND (conformation OR conformational): 11 applications
aclm/fluorescence AND aclm/(protein OR prion) AND aclm/(detect OR detects OR detection OR detecting OR detected): 1004 applications
aclm/fluorescence AND aclm/(prion) AND aclm/(detect OR detects OR detection OR detecting OR detected): 15 applications
aclm/fluorescence AND (diagnostic or diagnose or diagnosis or diagnosing) and prion andnot aclm/prion ANDNOT aclm/(detect OR detects OR detection OR detecting OR detected): 8 applications
aclm/(conformation OR conformational) AND (prion OR protein) AND folding AND (detect OR detects OR detection OR detecting OR detected OR detector OR detective): 315 applications
aclm/(conformation OR conformational) AND (prion OR protein) AND folding AND (detect OR detects OR detection OR detecting OR detected OR detector OR detective): 322 applications
aclm/(conformation OR conformational) AND (prion) AND folding AND (detect OR detects OR detection OR detecting OR detected OR detector OR detective): 38 applications
aclm/(conformation OR conformational) AND (prion OR protein) AND folding AND (detect OR detects OR detection OR detecting OR detected OR detector OR detective): 322 applications

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PATENTS from USPTO website:

pyrene AND excimer AND (conformation OR conformational): 44 patents
prion AND pyrene AND excimer AND (conformation OR conformational): 0 patents
aclm/protein AND pyrene AND excimer AND (conformation OR conformational): 5 patents
protein ANDNOT aclm/protein AND pyrene AND excimer AND (conformation OR conformational): 23 patents
aclm/fluorescence AND aclm/(protein OR prion) AND (conformation OR conformational): 191 patents
aclm/fluorescence AND aclm/(protein OR prion) AND aclm/(detect OR detects OR detection OR detecting OR detected): 402 patents
aclm/fluorescence AND aclm/prion AND aclm/(detect OR detects OR detection OR detecting OR detected): 2 patents
aclm/fluorescence AND prion ANDNOT aclm/prion AND aclm/(detect OR detects OR detection OR detecting OR detected): 13 patents
aclm/fluorescence AND (diagnostic OR diagnose OR diagnosis OR diagnosing) AND prion ANDNOT aclm/prion) ANDNOT aclm/(detect OR detects OR detection OR detecting OR detected): 3 patents
aclm/(conformation OR conformational) AND (prion OR protein) AND folding AND (detect OR detects OR detection OR detecting OR detected OR detector OR detective): 141 patents

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EPO website :
prion and (conformation or conformational) in the title or abstract : 29 hits
prion AND (detect or detection or detects or detected) in the title or abstract : 162 hits

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Keyword Search Terms

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=> fil dgene
=> run getseq VVAGAAAAGAMHKM/sqsp  :  3 hits
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POTENTIALLY RELEVANT REFERENCES IDENTIFIED IN PRIOR ART SEARCH
10/728,246

Patent Publications:

USP 6,399,314 to Krishnamurthy

JP 2004-155688 to Numao

Non-Patent Publications:

Salmona, M., *et al.*, "Molecular determinants of the physicochemical properties of a critical prion protein region comprising residues 106-126", *Biochem. J.* **342**:207-214, 1999.

Summary of Currently Claimed Invention:

The present invention is directed to conformational probes that change conformation from alpha-helix and/or random coil to beta-sheet upon contact with a beta sheet structure in a target protein. In embodiments, the invention is a method for detecting β-sheet conformation of proteins or prions in a sample. The method comprises 1) reacting the sample with one or more α-helix or random coil conformational probes that interact with β-sheet conformation proteins or prions in the sample and thereby undergo a conformational conversion to a predominantly β-sheet conformation and form detectable aggregates with the β-sheet conformation proteins or prions in the sample, and 2) detecting levels of detectable aggregates. (See, for example, current claim 1.) In embodiments, the method is a method of diagnosing whether a subject suffers from or is predisposed to a disease associated with conformationally altered proteins or prions, in which the method comprises obtaining a sample from the subject and performing the method of the invention discussed above. (See, for example, current claim 60.) In other embodiments, the present invention is a kit for performing the method. (See, for example, present claim 33.) Further, in embodiments, the present invention is a conformational probe, such as one for practicing the methods of the invention. In embodiments, the probe comprises a first and third portion that are identical in sequence to a peptide sequence of a target protein that can assume a β-sheet conformation, and a second portion that is between the first and third portions, and which contains 1-10 residues, one of which is a proline. (See, for example, current claim 89.) In embodiments, the probe comprises residues of the Abeta protein of Alzheimer's Disease (see, for example, claim 104).

Summary of References Identified and Discussion of Patentability of Current Claims:

I. U.S. Patent No. 6,399,314 to Krishnamurthy discloses assays for detecting the amyloid protein of Alzheimer's Disease (AD), and in particular, the aggregation state (misfolded/aggregated vs. properly folded/soluble) of the protein. (See the '314 patent at col. 2, lines 27-48, for example.) The methods include binding an amyloid-specific spectroscopic probe (*i.e.,* Congo red) or a fluorophore-labeled amyloid peptide probe to an amyloid protein in a sample, and detecting the bound probe or peptide by its increased fluorescence when bound to the misfolded protein as compared to the properly folded protein. (See the '314 patent at col. 2, lines 39-67; col. 5, lines 20-63; col. 6, lines 33-60; and col. 7, lines 40-52, for example.) As the present claims relate to binding of peptide probes to conformationally altered or misfolded proteins, the disclosure of the '314 patent relating to binding of Congo red, which is not a peptide probe, is not relevant.

With regard to peptide probes, the '314 patent discloses that the method of the invention comprises adding a fluorophore-labeled amyloid peptide probe to an amyloid polypeptide mixture in a given aggregation state (already misfolded or already properly folded), and determining the relative spectroscopic properties of the probe. (See the '314 patent at col. 11, lines 30-35.) In certain embodiments, the probe may be added prior to the amyloid polypeptide achieving its final aggregation state. (See the '314 patent at col. 7, lines 31-39, for example.) Depending on the aggregation state and level of β-sheet structure of the amyloid polypeptide, the fluorophore-labeled amyloid peptide probe will show different properties (*e.g.,* higher UV emission when bound to misfolded/aggregated/ high β-sheet amyloid polypeptide, different far-UV CD spectra). (See the '314 patent at col. 6, lines 33-60, for example.)

In contrast to the presently claimed invention, which utilizes probes that undergo a conformational shift from alpha-helix and/or random coil to beta-sheet, at no point does the '314 patent disclose or suggest that the amyloid polypeptide changes the structure of the fluorophore-labeled amyloid peptide probe to convert it from a predominantly α-helix and/or random coil conformation to a predominantly β-sheet conformation. Thus, the '314 patent is not anticipatory art under 35 U.S.C. § 102 against the currently pending claims. Because the '314 patent does not suggest this concept or motivate one to achieve this concept, it likewise is not prior art against the currently pending claims under 35 U.S.C. § 103.

In view of the failure of the '314 patent to disclose or suggest a conformational probe according to the present claims, much less use of such a conformational probe to detect the presence of β-sheet conformation of proteins, the '314 patent fails to anticipate or render obvious the presently claimed invention.

II. Based on the English translation of Japanese patent publication JP 2004-155688 to Numao (attached), this patent publication discloses synthetic peptides having molecular chaperone activity (see '688 patent publication at first paragraph, "Problems to be Solved by the Invention"). The peptides comprise defined amino acid sequences and have peptide decarboxylation activity on oxalo-acetate (see '688 patent publication at second paragraph, "Means to Solve the Problem" and paragraph [0008]). The amino acid sequences are taken from, or derived from, sequences known in the art as being present in the prion protein at the region known to undergo conversion from alpha-helix or random coil in the normal cellular protein to

beta-sheet in the infectious protein. The peptides are said to have pharmaceutical properties (see, for example, paragraphs [0010] - [0021], for example).

While the '688 patent publication discusses the potential effects of peptides drawn from the same region of a prion protein as peptide probes of the current invention can be drawn, it fails to teach and/or suggest the presently claimed invention. More specifically, in contrast to the presently claimed invention, which uses probes to detect β-sheet conformation proteins or prions by detecting conversion of the conformation of the conformational probe from alpha-helix and/or random coil to beta-sheet, at no point does the '688 patent publication disclose or suggest this concept. Rather, at its most relevant, it discloses peptides taken from a region of a prion protein that is similar to the region serving as the basis for some peptide sequences for embodiments of the present invention, and suggests that those peptides can bind to proteins in a sample and protect them from decarboxylation. It does not, however, disclose or suggest that those peptides undergo any conformational change, or that such a change (if it were to occur) could be monitored to provide a method of detecting conformationally altered or misfolded proteins. Thus, the '688 patent publication is not anticipatory art under 35 U.S.C. § 102 against the currently pending claims. Furthermore, because the '688 patent publication does not suggest this concept or motivate one to achieve this concept, it is not prior art against the currently pending claims under 35 U.S.C. § 103.

III. Salmona *et al.* discloses the characterization of residues involved in conversion of the prion protein from a normal cellular protein to an infectious, misfolded protein (see Salmona abstract; page 208, first full paragraph). To do so, Salmona *et al.* made various substitutions in the prion protein sequence at residues 106-126, and tested the resulting peptides for alpha-helical characteristics (see Salmona, Experimental section, pages 208-209). In the Salmona *et al.* discloses that the peptides were capable of self-aggregation in various environments, regardless of their overall conformation (*i.e.,* alpha-helical or beta-sheet). However, self-aggregation was found to occur more readily for alpha-helical peptides (*e.g.*, peptides at pH 7.0). Salmona *et al.* propose that aggregates are formed in an anti-parallel fashion with a β-pleated sheet structure. (See Salmona *et al.* at page 213, first full paragraph and Figure 4).

With regard to patentability of the present invention over Salmona *et al.*, it is submitted that Salmona *et al.* does not disclose or suggest conformational probes, or use of such probes to detect the presence of β-sheet conformation of proteins or prions in a sample. Rather, Salmona *et al.* is completely focused on determination of residues that are involved in conversion of alpha-helical peptide structures to beta-sheet structures. Thus, Salmona *et al.* fails to teach and/or suggest the presently claimed invention. At its most relevant, it discloses that prion peptides comprising residues from the 106-126 region can self-aggregate, and that peptides that have an alpha-helical structure are more prone to do so. This is in contrast to the presently claimed invention, which utilizes probes that can aggregate upon conversion from α-helical or random coil structures to β-sheet structures.

Furthermore, Salmona *et al.* is completely silent with regard to conversion of an alpha-helical and/or random coil structure in a peptide to a predominantly beta-sheet structure upon contact of the peptide with a peptide or protein having a beta-sheet structure. Furthermore, it does not disclose that such a conversion should be monitored to provide a method of detecting conformationally altered or misfolded proteins.

Thus, Salmona *et al.* is not anticipatory art under 35 U.S.C. § 102 against the currently pending claims. Furthermore, because Salmona *et al.* does not suggest this concept or motivate one to achieve this concept, it is not prior art against the currently pending claims under 35 U.S.C. § 103.

(19)日本国特許庁(JP)　(12)公開特許公報(A)　(11)特許出願公開番号
特開2004-155688
(P2004-155688A)
(43)公開日　平成16年6月3日(2004.6.3)

(51)Int.Cl.[7]	F I		テーマコード（参考）
C07K 7/08	C07K 7/08	ZNA	2G045
A61K 38/00	A61P 25/28		4C084
A61P 25/28	A61P 43/00	111	4H045
A61P 43/00	G01N 33/15	Z	
G01N 33/15	G01N 33/50	Z	

審査請求 未請求　請求項の数 10　OL　（全 22 頁）　最終頁に続く

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(22)出願日　平成14年11月5日(2002.11.5)
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(32)優先日　平成14年4月30日(2002.4.30)
(33)優先権主張国　日本国(JP)
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(32)優先日　平成14年7月10日(2002.7.10)
(33)優先権主張国　日本国(JP)
(31)優先権主張番号　特願2002-268260(P2002-268260)
(32)優先日　平成14年9月13日(2002.9.13)
(33)優先権主張国　日本国(JP)

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Fターム(参考)　2G045 AA40 BB51 DA20 DA36 FB01 GC10
4C084 AA02 AA06 BA01 BA08 BA18 BA23 CA59 DC50 NA14 ZA152 ZA162 ZA182 ZC022
4H045 AA10 AA30 BA16 EA20 EA50 FA10

(54)【発明の名称】シャペロン活性を有する合成ペプチド、脱炭酸活性の測定方法、伝達性海綿状脳症用薬剤及びその探索方法

(57)【要約】
【課題】分子シャペロン活性を有する新規な合成ペプチドや伝達性海綿状脳症用薬剤及びその探索方法等を提供する。
【解決手段】アミノ酸配列Val－Pro－Val－Ala－Pro－Gly－Ala－Pro－Ala－Ala－Pro－Ala－X_1（X_1はASP又はGluである。）を少なくとも一部に有するペプチド、又はIle－Ser－X_2－Gly－Ser－Gly－X_3－Thr－Trp－Ser－Asn－X_4－Tyr（X_2、X_3及びX_4はASP、Glu又はArgである。）を少なくとも一部に有するペプチドを、プリオン蛋白質のアミノ酸配列由来のLys－Thr－Asn－Met－Lys－His－Met－Ala－Gly－Ala－Ala－Ala－Ala－Gly－Ala－Val－Val－Gly－Gly－Leu－Glyを少なくとも一部に有するペプチドに加えることによって、後者のペプチドの脱炭酸活性を観測することができる。
【選択図】　なし

【特許請求の範囲】
【請求項１】
下記のアミノ酸配列
Ｖａｌ－Ｐｒｏ－Ｖａｌ－Ａｌａ－Ｐｒｏ－Ｇｌｙ－Ａｌａ－Ｐｒｏ－Ａｌａ－Ａｌａ－Ｐｒｏ－Ａｌａ－Ｘ$_1$（Ｘ$_1$はＡＳＰ又はＧｌｕを表す。）を少なくとも一部に有することを特徴とするシャペロン活性を有する合成ペプチド。
【請求項２】
下記のアミノ酸配列
Ｉｌｅ－Ｓｅｒ－Ｘ$_2$－Ｇｌｙ－Ｓｅｒ－Ｇｌｙ－Ｘ$_3$－Ｔｈｒ－ＴｒＰ－Ｓｅｒ－ＡＳｎ－Ｘ$_4$－Ｔｙｒ又は
Ｔｙｒ－Ｘ$_4$－ＡＳｎ－Ｓｅｒ－ＴｒＰ－Ｔｈｒ－Ｘ$_3$－Ｇｌｙ－Ｓｅｒ－Ｇｌｙ－Ｘ$_2$－Ｓｅｒ－Ｉｌｅ
（Ｘ$_2$、Ｘ$_3$及びＸ$_4$はＡＳＰ、Ｇｌｕ又はＡｒｇである。）を少なくとも一部に有することを特徴とするシャペロン活性を有する合成ペプチド。
【請求項３】
前記アミノ酸配列のＮ末端がＮＨ$_2$又はＮＨＣＯＣＨ$_3$であり、Ｃ末端がＣＯＯＨ又はＣＯＮＨ$_2$であることを特徴とする請求項１又は２に記載のシャペロン活性を有する合成ペプチド。
【請求項４】
下記のアミノ酸配列
Ｉｌｅ－Ｓｅｒ－Ｘ$_2$－Ｇｌｙ－Ｓｅｒ－Ｇｌｙ－Ｘ$_3$－Ｔｈｒ－ＴｒＰ－Ｓｅｒ－ＡＳｎ－Ｘ$_4$－Ｔｙｒ又は
Ｔｙｒ－Ｘ$_4$－ＡＳｎ－Ｓｅｒ－ＴｒＰ－Ｔｈｒ－Ｘ$_3$－Ｇｌｙ－Ｓｅｒ－Ｇｌｙ－Ｘ$_2$－Ｓｅｒ－Ｉｌｅ
（Ｘ$_2$、Ｘ$_3$及びＸ$_4$はＡＳＰ、Ｇｌｕ又はＡｒｇである。）を少なくとも一部に有するペプチドを、トリフロロエタノールとオキザロアセテートを含む緩衝液に加える工程を有することを特徴とする脱炭酸活性の測定方法。
【請求項５】
プリオン蛋白質のアミノ酸配列由来の下記のアミノ酸配列
ＬｙＳ－Ｔｈｒ－ＡＳｎ－Ｍｅｔ－ＬｙＳ－ＨｉＳ－Ｍｅｔ－Ａｌａ－Ｇｌｙ－Ａｌａ－Ａｌａ－Ａｌａ－Ａｌａ－Ｇｌｙ－Ａｌａ－Ｖａｌ－Ｖａｌ－Ｇｌｙ－Ｇｌｙ－Ｌｅｕ－Ｇｌｙ又は
Ｇｌｙ－Ｌｅｕ－Ｇｌｙ－Ｇｌｙ－Ｖａｌ－Ｖａｌ－Ａｌａ－Ｇｌｙ－Ａｌａ－Ａｌａ－Ａｌａ－Ａｌａ－Ｇｌｙ－Ａｌａ－Ｍｅｔ－ＨｉＳ－ＬｙＳ－Ｍｅｔ－ＡＳｎ－Ｔｈｒ－ＬｙＳ
を少なくとも一部に有するペプチドを、請求項１乃至３のいずれか１項に記載の合成ペプチドとオキザロアセテートを含む緩衝液に加える工程を有することを特徴とする脱炭酸活性の測定方法。
【請求項６】
前記アミノ酸配列のＮ末端をＮＨ$_2$又はＮＨＣＯＣＨ$_3$とし、Ｃ末端をＣＯＯＨ又はＣＯＮＨ$_2$とすることを特徴とする請求項４又は５に記載の脱炭酸活性の測定方法。
【請求項７】
請求項１乃至３のいずれか１項に記載の合成ペプチドを用いたことを特徴とする伝達性海綿状脳症用薬剤。
【請求項８】
プリオン蛋白質のアミノ酸配列由来の下記のアミノ酸配列
ＬｙＳ－Ｔｈｒ－ＡＳｎ－Ｍｅｔ－ＬｙＳ－ＨｉＳ－Ｍｅｔ－Ａｌａ－Ｇｌｙ－Ａｌａ－Ａｌａ－Ａｌａ－Ａｌａ－Ｇｌｙ－Ａｌａ－Ｖａｌ－Ｖａｌ－Ｇｌｙ－Ｇｌｙ－Ｌｅｕ－Ｇｌｙ又は
Ｇｌｙ－Ｌｅｕ－Ｇｌｙ－Ｇｌｙ－Ｖａｌ－Ｖａｌ－Ａｌａ－Ｇｌｙ－Ａｌａ－Ａｌａ－

Ａｌａ－Ａｌａ－Ｇｌｙ－Ａｌａ－Ｍｅｔ－Ｈｉｓ－Ｌｙｓ－Ｍｅｔ－Ａｓｎ－Ｔｈｒ－Ｌｙｓ
を少なくとも一部に有するペプチドを用いることを特徴とする伝達性海綿状脳症用薬剤の探索方法。
【請求項９】
前記アミノ酸配列のＮ末端をNH_2又は$NHCOCH_3$とし、Ｃ末端をＣＯＯＨ又は$CONH_2$とすることを特徴とする請求項８に記載の伝達性海綿状脳症用薬剤の探索方法。
【請求項１０】
塩基性アミノ酸リジン残基を少なくとも１個含む両親媒性アミノ酸配列を用いることを特徴とする伝達性海綿状脳症用薬剤の探索方法。
【発明の詳細な説明】
【０００１】
【発明の属する技術分野】
本発明は、プリオン蛋白質の部分アミノ酸配列（セグメント）に対してシャペロン活性を有する新規な合成ペプチド、脱炭酸活性の測定方法、伝達性海綿状脳症用薬剤及びその探索方法に関する。
【０００２】
【従来の技術】
伝達性海綿状脳症（クロイツフェルト・ヤコブ病、スクレイピー、牛海綿状脳症など）の治療薬の開発には、社会からの強力な要請がある。これまでに幾つかの低分子化合物が開発されてきているが、効力性と副作用の点から、より優れた活性をもつ化合物の開発が望まれている（Ｋ．Ｔ．Ａｄｊｏｕ ｅｔ ａｌ．，ＣＮＳ Ｄｒｕｇｓ １０．８３－８９（１９９８））。
【０００３】
伝達性海綿状脳症と高分子シャペロン（Ｐｒｏｔｅｉｎ Ｘ）との関連性は既に指摘されているが、高分子シャペロンの単離同定には到っていない（Ｆ．Ｅ．Ｃｏｈｅｎ ＆ Ｓ．Ｂ．Ｐｒｕｓｉｎｅｒ．Ａｎｎｕ．Ｒｅｖ．Ｂｉｏｃｈｅｍ．，６７．７９３－８１９（１９９８））。但し、１３個のアミノ酸残基からなる合成ペプチド（ｉＰｒＰ１３）によるヒト（又はマウス）感染性プリオン蛋白質（PrP^{Sc}）の正常プリオン蛋白質（PrP^{C}）への変換が既に報告されている（Ｃ．Ｓｏｔｏ ｅｔ ａｌ．，Ｌａｎｃｅｔ ３５５，１９２－１９７（２０００），Ｃ．Ｓｏｔｏ ｅｔ ａｌ．，Ｂｉｏｃｈｅｍ．ＢｉｏＰｈｙｓ．Ｃｈｅｍ．Ｃｏｍｍｕｎ．，２２６，６７２－６８０（１９９６））。
【０００４】
所望の分子シャペロン活性を有する短鎖ペプチド又は低分子有機化合物を開発するためには、生物活性を有する既存のアミノ酸配列中のアミノ酸残基を他のアミノ酸残基に置換するという方法や自然界からのランダムスクリーニング方法が採用される。例えば、前者の方法では、その配列中のアミノ酸残基の親媒性に着目して、似たような性質をもつほかのアミノ酸残基と入れ替える。また、Ｎ末端のアセチル化、Ｃ末端のアミド化、あるいはペプチド結合（－ＣＯＮＨ－）を$-CH_2-NH-$、$-CH=CH-$、$-NHCO-$のように変換したり、適当な位置に２つのシステイン残基を導入して直線型から環状型にしたり、あるいはその逆方向（環状型から直線型）へ変換したり、またＬ－アミノ酸残基をＤ－アミノ酸残基と交換したりする改変方法もある。更に、新しい１つの合成方法として、天然型アミノ酸配列の逆配列合成法もある（Ｂ－Ｌ．Ｌｉｅ ｅｔ ａｌ．，Ｂｉｏｌ．Ｐｈａｒｍ．Ｂｕｌｌ．，１９．１６０２－１６０６（１９９６））。
【０００５】
そのような既知手段の中で、Ｓｏｔｏ らはｉＰｒＰ１３の逆配列ペプチドに関する分子シャペロン活性を全く言及していない（Ｃ．Ｓｏｔｏ ｅｔ ａｌ．，Ｌａｎｃｅｔ ３５５，１９２－１９７（２０００），Ｃ．Ｓｏｔｏ ｅｔ ａｌ．，Ｂｉｏｃｈｅｍ．ＢｉｏＰｈｙｓ．Ｃｈｅｍ．Ｃｏｍｍｕｎ．，２２６，６７２－

６８０（１９９６））。

【０００６】

感染性プリオン蛋白質（ＰｒＰ^{Sc}）と伝達性海綿状脳症との関連性は多くの研究者に依って議論されているが、正常なプリオン蛋白質（ＰｒＰ^{C}）については、銅結合蛋白質やストレス応答蛋白質以外の機能は不明である。但し、プリオン蛋白質の部分ペプチドがトリフロロエタノール存在下、オキザロアセテートに対して脱炭酸活性を発現するかもしれないことが推測されているが実証されていない（特開２００２－２２７３６号公報）。

【０００７】

【発明が解決しようとする課題】

本発明の目的は、分子シャペロン活性を有する新規なシャペロン活性を有する合成ペプチド、脱炭酸活性の測定方法、伝達性海綿状脳症用薬剤及びその探索方法を提供することである。

【０００８】

【課題を解決するための手段】

本発明者は鋭意検討した結果、プリオン蛋白質のアミノ酸配列由来のアミノ酸配列R^4ＮＨ－ＬｙＳ－Ｔｈｒ－ＡＳｎ－Ｍｅｔ－ＬｙＳ－ＨｉＳ－Ｍｅｔ－Ａｌａ－Ｇｌｙ－Ａｌａ－Ａｌａ－Ａｌａ－Ａｌａ－Ｇｌｙ－Ａｌａ－Ｖａｌ－Ｖａｌ－Ｇｌｙ－Ｇｌｙ－Ｌｅｕ－Ｇｌｙ－ＣＯＲ^5（配列表中の配列番号１３）又はR^4ＮＨ－Ｇｌｙ－Ｌｅｕ－Ｇｌｙ－Ｇｌｙ－Ｖａｌ－Ｖａｌ－Ａｌａ－Ｇｌｙ－Ａｌａ－Ａｌａ－Ａｌａ－Ａｌａ－Ｇｌｙ－Ａｌａ－Ｍｅｔ－ＨｉＳ－ＬｙＳ－Ｍｅｔ－ＡＳｎ－Ｔｈｒ－ＬｙＳ－ＣＯＲ^5（配列表中の配列番号１４）（R^4は水素原子又はアセチル基であり、R^5はＯＨ又はNH_2である。）で表されるアミノ酸配列の何れか１つが、トリフロロエタノール（ＴＦＥ）存在下で、オキザロアセテートの脱炭酸活性を促進することを見出し、更にそのアミノ酸配列はアミノ酸配列R^1ＮＨ－Ｖａｌ－Ｐｒｏ－Ｖａｌ－Ａｌａ－Ｐｒｏ－Ｇｌｙ－Ａｌａ－Ｐｒｏ－Ａｌａ－Ａｌａ－Ｐｒｏ－Ａｌａ－X_1－ＣＯＲ^2（配列表中の配列番号１０）（X_1はＡＳＰ又はＧｌｕであり、R^1は水素原子又はアセチル基であり、R^2はＯＨ又はNH_2である。）で表されるペプチドか、又はR^3ＮＨ－Ｉｌｅ－Ｓｅｒ－X_2－Ｇｌｙ－Ｓｅｒ－Ｇｌｙ－X_3－Ｔｈｒ－ＴｒＰ－Ｓｅｒ－ＡＳｎ－X_4－ＴｙＲ－ＣＯＲ^4（配列表中の配列番号１１）若しくはR^3ＮＨ－ＴｙＲ－X_4－ＡＳｎ－Ｓｅｒ－ＴｒＰ－Ｔｈｒ－X_3－Ｇｌｙ－Ｓｅｒ－Ｇｌｙ－X_2－Ｓｅｒ－Ｉｌｅ－ＣＯＲ^4（配列表中の配列番号１２）（X_2、X_3及びX_4はＡＳＰ、Ｇｌｕ又はＡｒｇであり、R^1は水素原子又はアセチル基であり、R^2はＯＨ又はNH_2である。）で表されるペプチドのうち何れか１つのペプチド又はクロルプロマジンを加えると、ＴＦＥ非存在下でも、オキザロアセテートの脱炭酸活性を促進することを見出し、本発明を完成させた。

【０００９】

更に好ましい態様としては、X_1はＡＳＰ、X_2、X_3及びX_4はＡＳＰ又はＡｒｇである。

【００１０】

また、本願発明に係る伝達性海綿状脳症用薬剤の探索方法は、上記のいずれかの合成ペプチドを用いるか、又はカルシトニン等の塩基性アミノ酸リジン残基を少なくとも１個含む両親媒性アミノ酸配列を用いることを特徴とする。

【００１１】

【発明の実施の形態】

本発明のペプチドは活性化エステル法、混合酸無水物法、アジド法などのＣ端活性化法、カルボジミドなどのカップリング法、Ｎ－カルボキシ無水物（ＮＣＡ）法、酸化還元法あるいは固相合成法等の方法により合成することができる。

【００１２】

本発明のペプチドの有効成分として含む分子シャペロン剤においては該ペプチドに代えてあるいは該ペプチドと共に、上記のペプチドの生理学的に許容される塩を有効成分として含んでいてもよく、生理学的に許容される塩としてはアルカリ、無機酸または有機酸、例

えば水酸化ナトリウム、水酸化カルシウム、水酸化マグネシウム、水酸化カリウム、塩酸、硫酸、燐酸、酢酸、クエン酸、酒石酸、乳酸、オレイン酸、フマール酸等との塩を挙げることができる。

【００１３】

本発明におけるペプチドまたはその塩は治療または予防のための経口的あるいは非経口的に投与することができる。

【００１４】

経口投与剤としては散剤、　粒剤、カプセル剤、錠剤などの固形製剤あるいはシロップ剤、エリキシル剤などの液状製剤とすることができる。また、非経口投与剤としては注射剤、直腸投与剤、皮膚外用剤、吸入剤とすることができる。これらの製剤は活性成分に薬学的に認容できる製造助剤を加えることにより常法に従って製造される。更に、公知の技術により持続性製剤とすることも可能である。

【００１５】

経口投与用の固形製剤を製造するには活性成分と賦形剤、例えば乳糖、澱粉、結晶セルロース、乳酸カルシウム、メタケイ酸アルミン酸マグネシウム、無水ケイ酸などと混合して散剤とするか、更に必要に応じて白糖、ヒドロキシプロピルセルロース、ポリビニルピロリドンなどの結合剤、カルボキシメチルセルロース、カルボキシメチルセルロースカルシウムなどの崩壊剤などを加えて湿式または乾式造粒して　粒剤とする。錠剤を製造するにはこれらの散剤及び　粒剤をそのままあるいはステアリン酸マグネシウム、タルクなどの滑沢剤を加えて打錠すればよい。これらの　粒または錠剤はヒドロキシメチルセルロースフタレート、メタアクリル酸、メタアクリル酸メチルコポリマーなど腸溶性基剤で被覆して腸溶性製剤、あるいはエチルセルロース、カルナウバロウ、硬化油などで被覆して持続性製剤とすることもできる。また、カプセル剤を製造するには散剤又は　粒剤などの硬カプセルに充填するか、活性成分をグリセリン、ポリエチレングリコール、ゴマ油、オリーブ油などに溶解したのちゼラチン膜で被覆し軟カプセルとすることができる。

【００１６】

経口投与用の液状製剤を製造するには活性成分と白糖、ソルビトール、グリセリンなどの甘味剤とを水に溶かして透明なシロップ剤、更に精油、エタノールなどを加えてエリキシル剤とするか、アラビアゴム、トラガント、ポリソルベート８０、カルボキシメチルセルロースナトリウムなどを加えて乳剤または懸濁剤としてもよい。これらの液状製剤には所望により矯味剤、着色剤、保存剤などを加えてもよい。

【００１７】

注射剤を製造するには活性成分を必要に応じて塩酸、水酸化ナトリウム、乳酸、乳酸ナトリウム、リン酸一水素ナトリウム、リン酸二水素ナトリウムなどのＰＨ調整剤、塩化ナトリウム、ブドウ糖などの等張化剤とともに注射用蒸留水に溶解し、無菌ろ過してアンプルに充填するか、更にマンニトール、デキストリン、シクロデキストリン、ゼラチンなどを加えて真空下凍結乾燥し、用事溶解型の注射剤としてもよいし、活性成分にレシチン、ポリソルベート８０、ポリオキシエチレン硬化　麻子油などを加えて水中で乳化せしめ注射用乳剤とすることもできる。

【００１８】

直腸投与剤を製造するには活性成分及びカカオ脂、脂肪酸のモノ、ジ及びトリグリセリド、ポリエチレングリコールなどの坐剤用基剤とを加湿して溶融し、型に流し込んで冷却するか、活性成分をポリエチレングリコール、大豆油などに溶解したのちゼラチン膜で被覆すればよい。

【００１９】

皮膚外用剤を製造するには活性成分を白色ワセリン、ミツロウ、流動パラフィン、ポリエチレングリコールなどに加えて必要ならば加湿して練合し軟膏剤とするか、ロジン、アクリル酸アルキルエステル重合体などの粘着剤と練合したのちポリエチレンなどの不織布に展延してテープ剤としてもよい。

【００２０】

吸入剤を製造するには活性成分をフロンガスなどの噴射剤に溶解または分散して耐圧容器に充填しエアゾール剤としてもよい。

【００２１】

本発明のペプチドの投与量は伝達性海綿状脳症の患者（牛、羊などの動物も含め）の年齢、体重及び病態に依ってことなるが、通常一日当たり約１～５００ｍｇであり、１乃至数回に分けて投与することが望ましい。

【００２２】

以下に、本発明について、実施例に基づいて具体的に説明するが、本発明はこれらに限定されるものではない。また、本活性測定方法を全種プリオン蛋白質の全長アミノ酸配列に応用したり、アミノ酸親媒性などの分類法に関する既知知見を用いて蛋白質データベース等から本発明のシャペロン活性を有する合成ペプチドのアミノ酸配列と相同性の高い（４０％以上）配列を持つ蛋白質を探索し、その相同性の高いアミノ酸配列を含む該蛋白質のアミノ酸配列全体又は部分を同一目的（例えば、伝達性海綿状脳症の治療、予防又は検査薬の開発）に利用することも容易に類推でき、本発明範囲に含まれる。更に、本活性測定法は、伝達性海綿状脳症の治療、予防又は検査用の薬剤の探索方法にも利用できることは明白であり、その用途法が本実施例に限定されるものではない。

【００２３】

【実施例】

先ず、種々の合成ペプチドを準備した。本実施例に用いた合成ペプチドは下記表１に示すとおりである。

【００２４】

【表１】

配列番号	アミノ酸配列
1	GIGKFLKKAKKFAKAFVKILKK-$CONH_2$
2	LAKLLKALAKLLKK-$CONH_2$
3	KTNMKHMAGAAAAGAVVGGLG-COOH
4	GLGGVVAGAAAAGAMHKMNTK-COOH
5	DAPAAPAGPAVPV-COOH
6	VPVAPGAPAAPAD-COOH
7	ISRGSGRTWSNRY-COOH
8	ISDGSGDTWSNDY-COOH
9	CH_3CONH-KTNMKHMAGAAAAGAVVGGLG-COOH

【００２５】

配列番号１、２、３及び５に記載のアミノ酸配列は公知である（Ａ．Ｉｗａｈｏｒｉ ｅｔ ａｌ．．Ｂｉｏｌ．Ｐｈａｒｍ．Ｂｕｌｌ．２０．２６７－２７０（１９９７）；Ｋ．Ｊｏｈｎｓｓｏｎ ｅｔ ａｌ．．Ｎａｔｕｒｅ３６５．５３０－５３２（１９９３）；Ｇ．Ｆｏｒｉｏｎｉ ｅｔ ａｌ．Ｎａｔｕｒｅ ３６２．５４３－５４６（１９９３）；Ｃ．Ｓｏｔｏ ｅｔ ａｌ．．Ｌａｎｃｅｔ ３５５．１９２－１９７（２０００））。一方、配列番号４、６、７、８及び９に記載のアミノ酸配列は本発明範囲に含まれるものである。また、配列番号１、２、３、４及び９に記載のアミノ酸配列は、塩基性アミノ酸リジン残基を少なくとも１個含む両親媒性アミノ酸配列であり、このような両親媒性アミノ酸配列としては、他にカルシトニンが挙げられる。

【００２６】

次に、脱炭酸酵素活性測定を行った。この測定方法は以下のとおりである。２．９８ｍＭオキザロアセテート（１．７ｍｌ：５０ｍＭ ＭＯＰＳ．０．１５Ｍ ＮａＣｌ．ＰＨ７．５）とトリフロロエタノール（ＴＦＥ）（０．２ｍｌ）を分光器セルに加え、室温で、５分間 した。 後、その溶液に２．０ｍＭ濃度の測定用サンプルを０．１ｍｌ加えて反応溶液全量を２ｍｌとし、室温で、１分間 （Ｉｕｃｈｉ．ＨＳ－３Ｂ．回転スピード４）した。その後、 を停止し、分光器（ＵｌｔｒｏａｓＰｅｃ ３１００Ｐｒｏ．Ａｍｅｒｓｈａｍ ＢｉｏｓｃｉｅｎｅＳ ｃｏｒＰ．）で２８５ｎｍの吸光度を測定した。対照サンプルとしては２．０ｍＭ配列番号２（０．２ｍＭ）を用いた。

【００２７】
反応開始後、２５００秒後の配列番号２に記載のアミノ酸配列存在下での吸光度減少量ａを求めた。同様にして、配列番号２に記載のアミノ酸配列非存在下でのオキザロアセテートの吸光度減少量ｂを求めた。試験ペプチド（配列番号１、３、４、５、６、７及び８）についても同様にして吸光度減少量ｃを求め、下記の数式１により試験ペプチドの配列番号２に記載のアミノ酸配列に対する相対比活性を求めた。

【００２８】
【数１】

相対比活性＝（ｃ－ｂ）／（ａ－ｂ）

【００２９】
この測定結果を表２に示す。

【００３０】
【表２】

配列番号	相対比活性
1	0.32
2	1.00
3	0.12
4	0.12
5	0.02
6	0.04
7	NT
8	NT
9	NT

【００３１】
この結果より配列番号３及び４に記載のアミノ酸配列はＴＦＥ存在下脱炭酸活性を有していることが明らかとなった。

【００３２】
次に、合成ペプチドのシャペロン活性測定を行った。この測定方法は以下のとおりである。２．９８ｍＭオキザロアセテート（１．７ｍｌ：５０ｍＭ ＭＯＰＳ．０．１５Ｍ ＮａＣｌ．ＰＨ７．５）とＴＦＥ（０．２ｍｌ）を分光器セルに加え、室温で、５分間 した。 後、その溶液に２．０ｍＭ濃度の測定用サンプルを０．１ｍｌ加えて反応溶液全量を２ｍｌとし、室温で、１分間 （Ｉｕｃｈｉ．ＨＳ－３Ｂ．回転スピ

ード４）した。その後、　　を停止し、分光器（ＵｌｔｒｏａＳＰｅｃ　３１００Ｐｒｏ．　ＡｍｅｒＳｈａｍ　ＢｉｏＳｃｉｅｎｅＳ　ｃｏｒＰ．）で２８５ｎｍの吸光度を測定した。対照サンプルとしては２．０ｍＭ配列番号３（又は４）に記載のアミノ酸配列（０．２ｍＭ）を用いた。反応開始後、２５００秒後の配列番号３に記載のアミノ酸配列存在下での吸光度減少量ｄを求めた。同様にして、配列番号３に記載のアミノ酸配列非存在下でのオキザロアセテートの吸光度減少量ｅを求めた。

【００３３】

次に、上記操作のＴＦＥの代わりに試験ペプチド（配列番号５、６、７又は８）を０．２ｍｌ加え、室温で５分間　した。　後、その溶液に２．０ｍＭ濃度の測定用サンプル（配列番号３）を０．１ｍｌ加えて反応溶液全量を２ｍｌとし、室温で、１分間　（Ｉｕｃｈｉ．　ＨＳ－３Ｂ．　回転スピード４）した。　を停止後、分光器で２８５ｎｍの吸光度減少量ｆを測定した。下記の数式２により試験ペプチドの配列番号３（又は４）に記載のアミノ酸配列に対する相対比活性を求めた。

【００３４】

【数２】

相対比活性＝（ｆ－ｅ）／（ｄ－ｅ）

【００３５】

この測定結果を表３に示す。

【００３６】

【表３】

試料	相対比活性
配列番号３（200μM）＋TFE	1.00
配列番号４（200μM）＋TFE	1.00
配列番号３（200μM）＋配列番号５（100μM）	0.12
配列番号３（200μM）＋配列番号５（200μM）	0.38
配列番号３（200μM）＋配列番号６（100μM）	0.42
配列番号３（200μM）＋配列番号６（200μM）	0.44
配列番号３（200μM）＋配列番号７（100μM）	NT
配列番号３（200μM）＋配列番号７（200μM）	NT
配列番号３（200μM）＋配列番号８（100μM）	NT
配列番号３（200μM）＋配列番号８（200μM）	NT

【００３７】
この結果より、本発明のペプチド（配列番号６）はシャペロン活性を有していることが明らかとなった。
【００３８】
更に、他の方法により、合成ペプチドのシャペロン活性測定を行った。この測定方法は以下のとおりである。分光器セル中、２ｍＭ配列番号３（又は４）（０．２ｍｌ）、緩衝液（５０ｍＭ　ＭＯＰＳ、　０．１５Ｍ　ＮａＣｌ．　ＰＨ７．０）（１．４ｍｌ）の混合溶液に、２ｍＭ測定用サンプル（配列番号５．６．７．８）（０．２ｍｌ）を加え、室温で４８時間　（Ｉｕｃｈｉ．　ＨＳ－３Ｂ．　回転スピード４）した。　後、２．９８ｍＭオキザロアセテート（０．２ｍｌ）を加えて全量を２．０ｍｌとし、更に１分間　した。　を停止し、分光器（ＵｌｔｒａＳＰｅｃ　３１００Ｐｒｏ．　Ａｍｅｒｓｈａｍ　ＢｉｏＳｃｉｅｎｅＳ　ｃｏｒＰ．）で２８５ｎｍの吸光度減少量を反応開始後５０００秒間測定した。対照サンプルとしては、測定用サンプル非存在下、トリフロロエタノール（ＴＦＥ）存在下の配列番号３で測定した吸光度減少量を１．００とした。
【００３９】
この測定結果を表４に示す。
【００４０】
【表４】

試料	相対比活性
配列番号３（200μM）＋TFE（+）	1.00
配列番号３（200μM）＋TFE（-）	0.59
配列番号４（200μM）＋TFE（+）	0.70
配列番号４（200μM）＋TFE（-）	0.54
配列番号３（200μM）＋配列番号５（200μM）	0.62
配列番号３（200μM）＋配列番号６（200μM）	0.60
配列番号３（200μM）＋配列番号７（200μM）	0.65
配列番号３（200μM）＋配列番号８（200μM）	0.57

【００４１】
この結果より、本発明のペプチド（配列番号６及び７）はシャペロン活性を有していることが明らかとなった。
【００４２】
次に、上記の方法とは異なる方法により、脱炭酸活性測定を行った。この測定方法は以下のとおりである。分光器セル中、２ｍＭ配列番号９（０．２ｍｌ）、緩衝液（５０ｍＭ ＭＯＰＳ、０．１５ＭＮａＣｌ、ＰＨ７．０）（１．４ｍｌ）の混合溶液に、ＴＦＥ（０．２ｍｌ）を加え、室温で４８時間（Ｉｕｃｈｉ．ＨＳ－３Ｂ．回転スピード４）した。後、２．９８ｍＭオキザロアセテート（０．２ｍｌ）を加えて全量を２．０ｍｌとし、更に１分間した。を停止し、分光器（ＵｌｔｒａＳＰｅｃ ３１００Ｐｒｏ．Ａｍｅｒｓｈａｍ ＢｉｏＳｃｉｅｎｅＳ ｃｏｒＰ．）で２８５ｎｍの吸光度減少量を反応開始後５０００秒間測定した。対照サンプルとしては、測定用サンプル非存在下、トリフロロエタノール（ＴＦＥ）存在下の配列番号２で測定した吸光度減少量を１．００とした。
【００４３】
この測定結果を表５に示す。
【００４４】
【表５】

試料	活性	相対比活性
配列番号２（200μM）＋TFE（+）	0.195	1.00
配列番号２（200μM）＋TFE（-）	0.159	
配列番号９（200μM）＋TFE（+）	0.169	0.61
配列番号９（200μM）＋TFE（-）	0.147	

【００４５】
この結果より、配列番号９に記載のアミノ酸配列はＴＦＥ存在下で脱炭酸活性を有していることが明らかとなった。
【００４６】
次に、抗精神病薬等として使用されるクロルプロマジンのシャペロン活性測定を行った。この測定方法は以下のとおりである。分光器セル中、２ｍＭ配列番号９（０．２ｍｌ）、緩衝液（５０ｍＭ　ＭＯＰＳ．　０．１５ＭＮａＣｌ．　ＰＨ６．０）（１．４ｍｌ）の混合溶液に、２ｍＭ測定用サンプル（例えば、クロルプロマジン）を加え、室温で４８時間　（Ｉｕｃｈｉ．　ＨＳ－３Ｂ．　回転スピード４）した。　後、２．９８ｍＭオキザロアセテート（０．２ｍｌ）を加えて全量を２．０ｍｌとし、更に１分間　した。　を停止し、分光器（ＵｌｔｒａＳＰｅｃ　３１００Ｐｒｏ．　Ａｍｅｒｓｈａｍ　Ｂｉｏｓｃｉｅｎｅｓ　ｃｏｒＰ．）で２８５ｎｍの吸光度減少量を反応開始後５０００秒間測定した。対照サンプルとしては、配列番号９非存在下、探索用サンプル（クロルプロマジン）存在下で測定した吸光度減少量を１．００とした。
【００４７】
この測定結果を表６に示す。
【００４８】
【表６】

試料	相対比活性
ｸﾛﾙﾌﾟﾛﾏｼﾞﾝ（200μM）（pH6.0）	1.00
緩衝溶液中でのｵｷｻﾞﾛｱｾﾃｰﾄの自己分解（pH6.0）	1.00
配列番号９（200μM）+ｸﾛﾙﾌﾟﾛﾏｼﾞﾝ（200μM）（pH6.0）	1.31

【００４９】
この結果より、本発明のシャペロン活性測定法は伝達性海綿状脳症用治療薬としての低分子有機化合物を探索できることが明らかとなった。
【００５０】
【発明の効果】
上記ペプチドは分子シャペロン活性を有し、伝達性海綿状脳症の治療、予防又は検査薬としての用途を有する。
【００５１】
【配列表】

〈110〉 BioFrontier Institute INC.

〈120〉 Synthetic Peptide with Chaperone Activity

〈130〉 XY04651

〈140〉
〈141〉

〈150〉 JP 2002-128976
〈151〉 2002-4-30

〈150〉 JP 2002-200884
〈151〉 2002-7-10

〈150〉 JP 2002-268260
〈151〉 2002-9-13

〈160〉 14

〈170〉 PatentIn Ver. 2.1

〈210〉 1
〈211〉 22
〈212〉 PRT
〈213〉 Artificial Sequence

〈220〉
〈223〉 Synthetic Peptide

〈220〉
〈223〉 Topology is normal chain type

〈400〉 1
Gly Ile Gly Lys Phe Leu Lys Lys Ala Lys Lys Phe Ala Lys Ala Phe
1 5 10 15

Val Lys Ile Leu Lys Lys
20

〈210〉 2
〈211〉 14
〈212〉 PRT
〈213〉 Artificial Sequence

〈220〉
〈223〉 Synthetic Peptide

〈220〉
〈223〉 Topology is normal chain type

〈400〉 2
Leu Ala Lys Leu Leu Lys Ala Leu Ala Lys Leu Leu Lys Lys
1 5 10

〈210〉 3

〈211〉 21
〈212〉 PRT
〈213〉 Artificial Sequence

〈220〉
〈223〉 Synthetic Peptide

〈220〉
〈223〉 Topology is normal chain type

〈400〉 3
Lys Thr Asn Met Lys His Met Ala Gly Ala Ala Ala Ala Gly Ala Val
1 5 10 15

Val Gly Gly Leu Gly
20

〈210〉 4
〈211〉 21
〈212〉 PRT
〈213〉 Artificial Sequence

〈220〉
〈223〉 Synthetic Peptide

〈220〉
〈223〉 Topology is normal chain type

〈400〉 4
Gly Leu Gly Gly Val Val Ala Gly Ala Ala Ala Ala Gly Ala Met His
1 5 10 15

Lys Met Asn Thr Lys
20

〈210〉 5
〈211〉 13
〈212〉 PRT
〈213〉 Artificial Sequence

〈220〉
〈223〉 Synthetic Peptide

〈220〉
〈223〉 Topology is normal chain type

〈400〉 5
Asp Ala Pro Ala Ala Pro Ala Gly Pro Ala Val Pro Val
1 5 10

〈210〉 6
〈211〉 13
〈212〉 PRT
〈213〉 Artificial Sequence

〈220〉
〈223〉 Synthetic Peptide

〈220〉
〈223〉 Topology is normal chain type

〈400〉 6
Val Pro Val Ala Pro Gly Ala Pro Ala Ala Pro Ala Asp
1 5 10

〈210〉 7
〈211〉 13
〈212〉 PRT
〈213〉 Artificial Sequence

〈220〉
〈223〉 Synthetic Peptide

〈220〉
〈223〉 Topology is normal chain type

〈400〉 7
Ile Ser Arg Gly Ser Gly Arg Thr Trp Ser Asn Arg Tyr
1 5 10

〈210〉 8
〈211〉 13

〈212〉 PRT
〈213〉 Artificial Sequence

〈220〉
〈223〉 Synthetic Peptide

〈220〉
〈223〉 Topology is normal chain type

〈400〉 8
Ile Ser Asp Gly Ser Gly Asp Thr Trp Ser Asn Asp Tyr
1 5 10

〈210〉 9
〈211〉 21
〈212〉 PRT
〈213〉 Artificial Sequence

〈220〉
〈223〉 Synthetic Peptide

〈220〉
〈223〉 Topology is normal chain type

〈220〉
〈223〉 C-terminus is CH_3CONH

〈400〉 9

Lys Thr Asn Met Lys His Met Ala Gly Ala Ala Ala Ala Gly Ala Val
1 5 10 15

Val Gly Gly Leu Gly
20

〈210〉 10
〈211〉 13
〈212〉 PRT
〈213〉 Artificial Sequence

〈220〉
〈223〉 Synthetic Peptide

〈220〉
〈221〉 PEPTIDE
〈222〉 (13)
〈223〉 Xaa represents Asp or Glu

〈400〉 10
Val Pro Val Ala Pro Gly Ala Pro Ala Ala Pro Ala Xaa
1 5 10

〈210〉 11
〈211〉 13
〈212〉 PRT
〈213〉 Artificial Sequence

〈220〉
〈223〉 Synthetic Peptide

〈220〉
〈221〉 PEPTIDE
〈222〉 (3)
〈223〉 Xaa represents Asp, Glu or Arg

〈220〉
〈221〉 PEPTIDE
〈222〉 (7)
〈223〉 Xaa represents Asp, Glu or Arg

〈220〉
〈221〉 PEPTIDE
〈222〉 (12)
〈223〉 Xaa represents Asp, Glu or Arg

〈400〉 11
Ile Ser Xaa Gly Ser Gly Xaa Thr Trp Ser Asn Xaa Tyr
1 5 10

〈210〉 12
〈211〉 13
〈212〉 PRT
〈213〉 Artificial Sequence

〈220〉
〈223〉 Synthetic Peptide

〈220〉
〈221〉 PEPTIDE
〈222〉 (2)
〈223〉 Xaa represents Asp, Glu or Arg

〈220〉
〈221〉 PEPTIDE
〈222〉 (7)
〈223〉 Xaa represents Asp, Glu or Arg

〈220〉
〈221〉 PEPTIDE
〈222〉 (11)
〈223〉 Xaa represents Asp, Glu or Arg

〈400〉 12
Tyr Xaa Asn Ser Trp Thr Xaa Gly Ser Gly Xaa Ser Ile
1 5 10

〈210〉 13
〈211〉 21
〈212〉 PRT
〈213〉 Artificial Sequence

〈220〉

〈223〉 Synthetic Peptide

〈400〉 13
Lys Thr Asn Met Lys His Met Ala Gly Ala Ala Ala Ala Gly Ala Val
1 5 10 15

Val Gly Gly Leu Gly
20

〈210〉 14
〈211〉 21
〈212〉 PRT
〈213〉 Artificial Sequence

〈220〉
〈223〉 Synthetic Peptide

〈400〉 14
Gly Leu Gly Gly Val Val Ala Gly Ala Ala Ala Ala Gly Ala Met His
1 5 10 15

Lys Met Asn Thr Lys
20

フロントページの続き

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(43) [Publication Date of Unexamined Application]
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(43) [Publication Date of Unexamined Application]
2004-06-03
(54) [Title of Invention]
Synthetic Peptide Having Chaperone Activity, Method for Measuring Decarboxylation Thereof, Pharmaceutical For Transmissible Spongiform Encephalopathy, and Research Measurement Method Therefor
(51) [International Patent Classification, 7th Edition]
C07K7/08 A61K38/00 A61P25/28 A61P43/00 G01N33/15 G01N33/50 G01N33/68
[FI] C07K7/08 A61P25/28 A61P43/00111 G01N33/15Z G01N33/50Z G01N33/68 A61K37/02
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2G045 4C084 4H045
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2G045AA40 2G045BB51 2G045DA20 2G045DA36 2G045FB01 2G045GC 10
4C084AA02 4C084AA06 4C084BA 01 4C084BA 08 4C084BA 18 4C084BA 23
4C084CA59 4C084DC50 4C084NA14 4C084ZA152 4C084ZA162 4C084ZA182
4C084ZC022 4H045AA10 4H045AA30 4H045BA 16 4H045EA20 4H045EA50
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[Priority Date] 2002-04-30
[Priority Country] JP
[Priority Application Number] 2002200884
[Priority Date] 2002-07-10
[Priority Country] JP
[Priority Application Number] 2002268260
[Priority Date] 2002-09-13
[Priority Country] JP
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[Patent Attorney]
[Name] Kokubun Takayoshi

[Problems to be Solved by the Invention]
To provide a new synthetic peptide having a molecular chaperone activity, to provide a pharmaceutical for transmissible spongiform encephalopathy, to provide a method for researching the same, and the like.

[Means to Solve the Problems]
Amino acid sequence Val-Pro-Val-Ala-Pro-Gly-Ala-Pro-Ala-Ala-Pro-Ala-X(1) [where (X(1) is Asp or Glu] peptide, or Ile-Ser-X(2)-Gly-Ser-Gly-X(3)-Thr-Trp-Ser-Asn-X(4)-Tyr [where X(2), X(3) and X(4) are Asp , Glu or Arg]. Peptide at least in part adds to peptide which comprises Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly of the amino acid sequence derived from prion protein, such that decarboxylation activity of peptide of the latter can be observed.

[Selected Drawing] none

[Claims]

[Claim 1]
A synthetic peptide comprising the sequence: Val-Pro-Val-Ala-Pro-Gly-Ala-Pro-Ala-Ala-Pro-Ala-X(1) where X(1) is Asp or Glu, where the synthetic peptide possesses chaperone activity.

[Claim 2]
A synthetic peptide comprising the sequence: Ile-Ser-X(2)-Gly-Ser-Gly-X(3)-Thr-Trp-Ser-Asn-X(4)-Tyr or Tyr-X(4)-Asn-Ser-Trp-Thr-X(3)-Gly-Ser-Gly-X(2)-Ser-Ile, where X(2), X(3)and X(4) are Asp, Glu or Arg, where the synthetic peptide possesses chaperone activity.

[Claim 3]
The N-terminal portion of aforementioned amino acid sequence begining with NH2 or NHCOCH3, in Claim 1 or 2, with C-terminal being COOH or CONH2, and where the stated synthetic peptide possesses chaperone activity.

[Claim 4]
An amino acid sequence comprising: Ile-Ser-X(2)-Gly-Ser-Gly-X(3)-Thr-Trp-Ser-Asn-X(4)-Tyr or Tyr-X(4)-Asn-Ser-Trp-Thr-X(3)-Gly-Ser-Gly-X(2)-Ser-Ile where X(2), X(3) and X(4) are Asp, Glu or Arg, which may be used for purposes of a measurement method, to measure decarboxylation activity, when added to a buffer which includes trifluoro-ethanol and oxalo-acetate.

[Claim 5]
A peptide having an amino acid sequence derived from a prion protein, comprising: Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly or Gly-Leu-Gly-Gly-Val-Val-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Met-His-Lys-Met-Asn-Thr-Lys, which may be used for purposes of a measurement method, to measure decarboxylation activity when added to a buffer which includes synthetic peptide and oxalo acetate which stated peptide comprises at least in part, the peptides stated in the any one claim of Claims 1 through 3.

[Claim 6]
A measurement method for measuring decarboxylation activity, as stated in Claim 4 or 5, using peptide with a designated N- terminal of aforementioned amino acid sequence as being NH2 or NHCOCH3, and with designated C-terminal as being COOH or CONH2.

[Claim 7]
A pharmaceutical drug for transmissible spongiform encephalopathy, designated as the synthetic peptide stated in any one claim of Claims 1 through 3.

[Claim 8]
An amino acid sequence derived from the amino acid sequence prion protein, comprising the sequence Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly or Gly-Leu-Gly-Gly-Val-Val-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Met-His-Lys-Met-Asn-Thr-Lys, used for purposes of a research method for screening pharmaceutical drugs for transmissible spongiform encephalopathy, designating any peptide which possesses such sequence at least in part.

[Claim 9]
A research method for screening pharmaceutical drugs for transmissible spongiform encephalopathy, as stated in Claim 8, which peptide is designated to have an N-terminal of aforementioned amino acid sequence as being NH2 or NHCOCH3, and a C-terminal as being COOH or CONH2.

[Claim 10]
A research method for screening pharmaceutical drugs for transmissible spongiform encephalopathy, designating an amphipathic amino acid sequence which includes a basic amino acid such as a lysine residue as at least one of the amino acid residues.

[Description of the Invention]

[0001]
[Technological Field of Invention]
This invention relates to a pharmaceutical drug for transmissible spongiform encephalopathy and a research method for measuring decarboxylation activity of novel synthetic peptide which possesses chaperone activity, comprising a partial amino acid sequence of prion protein.

[0002] [Prior Art]
There is strong request from society for development of a treatment drug for transmissible spongiform encephalopathy such as Creutzfeldt-Jakob illness [sukureipii], or bovine spongiform encephalopathy. (K.T. Adjou et al., CNS Drugs 10:83-89 [1998]). Several low-molecular weight compounds have been developed so far, but it is desired that compounds with such activity have better effectiveness and fewer side effects.

[0003]
As described by F.E.Cohen and S.B.Prusiner [Annu. Rev. Biochem. 67:793-819 (1998)], transmissible spongiform encephalopathy may involve a polypeptide chaperone (protein X), or polymeric form of the chaperone [C. Soto et al., Lancet 355(9199):192-7 (2000); C. Soto et al., Biochem Biophys Res Commun. 226(3):672-80 (1996)]. Furthermore, conversion from normal form of human (or mouse) prion protein (PrPc) to the contagious form (PrPsc) is reported using a synthetic peptide (iPrP-13) consisting of 13 amino acid residues.

[0004]
In order to develop a short chain peptide of low molecular weight as an organic compound which possesses desired molecular chaperone activity, a random screening method was adopted, with substituting an amino acid residue within an existing amino acid sequence which possesses biological activity. With using methods from earlier studies, paying attention to characteristics of amino acid residues, such methods replaced an amino acid residue with another which has similar properties. In addition, there are also alterative methods using -CH(2)-NH-, -CH=CH-, -NHCO- to produce conversion by amidation reaction , to produce acetylation of the peptide bond (-CONH-) , or acetylation of the C-terminal or the N-terminal, or by introducing cysteine residues into suitable location , changing the linear structure to a cyclic structure , or from cyclic to linear type, in addition by exchanging L- amino acid residues with D- amino acid residues, or by constructing inverse sequence peptides. [B.L Lie et al., Biol Pharm Bull. 19(12):1602-6 (1996)].

[0005]
For example, as reported by C. Soto et al (op. cit.) such methods can be used to produce peptides that block conversion to the beta-sheet conformation.

[0006]
The relevance of contagious prion protein (PrPsc) to transmissible spongiform encephalopathy is argued by many researchers, but a precise connection with normal prion protein (PrPc) function other than via the stress response proteins remains unclear. However, from studies of synthetic peptides made from the prion protein using trifluoro-ethanol, it is presumed (although not proven) that such connection might reveal decarboxylation activity vis-a-vis the oxalo-acetate (Japan Unexamined Patent Publication 2002-22736 disclosure).

[0007]
[Problems to be Solved by the Invention]

An objective of this invention is to provide a compound for a method of measuring [or detecting] transmissible spongiform encephalopathy using a synthetic peptide, by measuring decarboxylation activity concomitant with molecular chaperone activity, and to provide a research method for same.

[0008]
[Means to Solve the Problems]
A peptide discovered by this inventor, as the result of diligent investigation, comprises the amino acid sequence R4NH-Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly-COR5 as derived from the amino acid sequence of prion protein (Sequence Number 13 in sequence table) or R4NH-Gly-Leu-Gly-Gly-Val-Val-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Met-His-Lys-Met-Asn-Thr-Lys-COR5 (Sequence Number 14 in sequence table) (where R4 denotes hydrogen atom or acetyl group , and R5 denotes OH or NH2) where either of these amino acid sequences is shown by using measures of trifluoro-ethanol (TFE), to determine whether decarboxylation activity of oxalo-acetate is promoted. Furthermore, as for amino acid sequence amino acid sequence R1NH-Val-Pro-Val-Ala-Pro-Gly-Ala-Pro-Ala-Ala-Pro-Ala-X(1)-COR2 (Sequence Number 10 in sequence table) (where X(1) denotes Asp or Glu , and where Rl denotes hydrogen atom or acetyl group , and where R2 denotes OH or NH2), or as for R3NH-Ile-Ser-X(2)-Gly-Ser-Gly-X(3)-Thr-Trp-Ser-Asn-X(4)-Tyr-COR4 (Sequence Number 11 in sequence table) or R3NH-Tyr-X(4)-Asn-Ser-Trp-Thr-X(3)-Gly-Ser-Gly-X(2)-Ser-Ile-COR4 (Sequence Number 12 in sequence table) (where X(2), X(3) and X(4) denotes Asp , Glu or Arg, and where Rl denotes hydrogen atom or acetyl group , and where R2 denotes OH or NH2). For any of the foregoing peptides, as compared with added chlopromazine, it can be determined whether decarboxylation activity of oxalo-acetate is promoted, which can be discovered even with the absence of TFE, by which discovery this invention was completed.

[0009]
Furthermore, X(1) may denote Asp , and X(2), X(3) and X(4) may denote Asp or Arg, as additional desirable embodiments.

[0010]
In addition, a research method for drugs for transmissible spongiform encephalopathy relates to the invention of this application, using any of the above-mentioned synthetic peptides, or designating that an amphipathic amino acid sequence as for example which occurs in calcitonin, with at least one or more basic amino acid lysine residues.

[0011]
[Embodiment of the Invention]
The synthesis of peptides according to this invention may be accomplished using an activated ester method , mixed acid anhydride method, azide method or other C-terminus activation method , carbodiimide or other coupling method, or an N-carboxy anhydride (NCA) method, with an oxidation and reduction method or solid phase synthesis method or other method.

[0012]
Replacing the said peptide as active ingredient with the peptide of this invention concerns a molecular chaperone agent which it includes, or which combines with said peptide , by means of which it is possible to include a physiologically acceptable salt of the above-mentioned peptide , as the active ingredient as physiologically acceptable salt, or alkali inorganic acid or organic acid , for example sodium hydroxide, calcium hydroxide, magnesium hydroxide, potassium hydroxide, hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid , citric acid , tartaric acid , lactic acid , oleic acid , fumaric acid, etc.

[0013]
The prescribed peptide or its salt may, according to this invention, be made for oral or parenteral delivery, and for treatment or prevention.

[0014]
The prescribed peptide can be made into a powder, granule, capsule, tablet or other solid preparation or syrup, elixir or other liquid state for formulation as an oral dosage agent. In addition, it can made injectable, for rectal administration, for an external skin preparation, for an inhalant, or for parenteral administration. These formulations following conventional methods, by adding manufacturing processes by which the active ingredient in pharmacological may be produced. Furthermore, it is also possible to make an increased retention formulation, with known technology.

[0015]

A solid preparation for oral dosage is produced, using active ingredient and a vehicle, for example lactose, starch, crystalline cellulose, calcium lactate, sodium metasilicate aluminate magnesium, anhydrous silicic acid, etc; and by mixing or making a powder , or with wet type or dry type granulation using sucrose [hidorokishi puropiru seruroosu], poly vinyl pyrrolidone chain or other binder [karuboki shimechiru seruroosu], including [karubokishi mechiruseruro osukarushiumu] or other disintegrating granulating agent to make tablets. These powder and granule pill-making processes must be done using magnesium stearate ** [or other substances], including talc or other lubricant. The granule or tablet sheath may be made with an enteric base , such as hydroxymethyl cellulose phthalate, methacrylic acid, methyl methacrylate copolymer, or made with other enteric sheath formulation, such as ethyl cellulose, carnauba wax, or hydrogenated oil to make an increased retention formulation. In addition, capsules produced may be filled with powder, or with granules or other hard capsules after melting active ingredient in glycerin, polyethylene glycol chain, sesame oil, olive oil etc. The sheath may also be made with gelatin film to make a soft capsule.

[0016]
A liquid state formulation for oral dosage is produced using the active ingredient and melting sucrose, sorbitol, glycerin or other sweetener in water, to makes an elixir of transparent syrup. Furthermore, by using an essential oil, with ethanol etc, it is possible to make an emulsion or suspension, by including gum arabic, traganth, polysorbate 80, [karubokishi mechiruseruro osunatoriumu] etc. These liquid state formulations may include desired flavoring, colorant, preservative etc

[0017]
An injectable form is produced with hydrochloric acid, sodium hydroxide, lactic acid, sodium lactate, sodium hydrogen phosphate **, or with sodium dihydrogen phosphate ** or with other pH adjustment agent, sodium chloride, fructose or other isotonic agent, which melts the active ingredient in injectable distilled water, produced using sterile filters, and filled ampoules, or by lyophilizing under vacuum. Furthermore, by using mannitol, dextrin, cyclodextrin, gelatin, etc it is possible to produce an injectable form of soluble type. An active ingredient emulsifying agent may include lecithin, polysorbate 80, polyoxyethylene hardening ** to make an injectable emulsion.

[0018]
A rectal administration agent is produced by humidifying active ingredient and cacao butter, aliphatic acid and base for triglyceride, polyethylene glycol chain or other suppository sink that is packed in type and cools, or after melting active ingredient in polyethylene glycol chain, soybean oil etc, with a sheath of gelatin film.

[0019]
An external skin preparation is produced by adding active ingredient to the white vaseline, beeswax, liquid paraffin, polyethylene glycol chain etc, humidifying or kneading the combination to make an ointment, or rosin , using alkyl acrylate ester polymer or other adhesive and kneading the combination after spreading/displaying or extending with a polyethylene or other nonwoven fabric.

[0020]
An inhalant is produced with active ingredient in freon gas or other propellant melting or dispersing, or filled in a pressure resistant vessel as an aerosol agent.

[0021]
A dose of peptide of this invention differs depending upon age, body weight and disease of patient (cattle, sheep or other animal including) of transmissible spongiform encephalopathy , but usually with per day approximately 1 to 500 mg , divided into one or several dosage times , as desirable to prescribe.

[0022]
Below, the invention is explained more concretely, on the basis of a Working Example, but not limited to this. In addition, protein which has amino acid sequence of synthetic peptide which applies this activity measurement method to the total length of an amino acid sequence of all kinds of prion protein, possesses chaperone activity according to the present invention, making use of known amino acid characteristics or other taxonomy and (40% or higher) homology, the claims of this invention include utilizing an amino acid sequence entirely or portion thereof to include an amino acid sequence where homology is high, for the same objectives (i.e., treatment, or prevention of transmissible spongiform encephalopathy or development of diagnostics). Furthermore, as for this activity measurement method , as for being able to utilize the method also for, prevention of transmissible spongiform encephalopathy or research method for a drug or diagnostic, it is not something where application method is limited in the this working example.

[0023]
[Working Example (s)]
First, various synthetic peptides were prepared.
Synthetic peptides used for this working example are shown in the below-mentioned Table 1.

[0024] [Table 1]

	SEQUENCE
1	GIGKFLKKAKKFAKAFVKILKK-CONH2
2	LAKLLKALAKLLKK-CONH2
3	KTNMKHMAGAAAAGAVVGGLG-COOH
4	GLGGVVAGAAAAGAMHKMNTK-COOH
5	DAPAAPAGPAVPV-COOH
6	VPVAPGAPAAPAD-COOH
7	ISRGSGRTWSNRY-COOH
8	ISDGSGDTWSNDY-COOH
9	CH3CONH-KTNMKHMAGAAAAGAVVGGLG-COOH

[0025]
As described in the publications of prior art [A. Iwahori et al., Biol Pharm Bull. 20(3):267-70 (1997); K. Johnsson et al., Nature 365(6446):530-2. (1993); G. Forloni et al., Nature 362(6420):543-6 (1993); C. Soto et al., Lancet 355(9199):192-7 (2000)] the amino acid sequences in Sequence Numbers 1, 2, 3 and 5 are public knowledge. Amino acid sequences in Sequence Numbers 4, 6, 7, 8 and 9 are included in claims of this invention. In addition, for amino acid sequence which are stated in Sequence Number 1,2,3,4 and 9, at least one amphipathic amino acid sequence may be included, such as exists in calcitonin, with basic amino acid lysine residues or other amino acid.

[0026]
Next, decarboxylase activity measurement was done. This measurement method is as follows. 2.98 mM oxalo acetate (1.7 ml ; 50mM MOPS, 0.15M NaCl, pH 7.5) with trifluoro-ethanol (TFE) (0.2 ml) by addition to a spectroscope cell, at room temperature, and with 5 min agitation. Adding 0.1 ml of measured sample of 2.0 mM concentration to solution of after stirring,, to obtain a reaction solution total amount of 2 ml, at room temperature, with 1 minute agitation (Iuchi, HS-3B, rotation speed 4). After that, churning was stopped, absorbance at 285 nm was measured with using a spectroscope (Ultroaspec3100pro, Amersham Bioscienescorp.). A sample of Sequence Number 2 (0.2 mM) was used as control sample.

[0027]
After starting the reaction, absorbance was reduced for an amino acid sequence as stated in Sequence Number 2, after 2500 seconds. Similarly, absorbance was reduced for oxalo-acetate using amino acid Sequence Number 2. Concerning test peptide (Sequence Number 1, 3, 4, 5, 6 , 7 and 8) absorbance was reduced similarly, with the relative ratio activity for amino acid sequences in Sequence Number 2 of test peptide with below-mentioned Mathematical Formula 1.

[0028]
[Mathematical Formula 1]

$$[\text{--------}] = (c-b) / (a-b)$$

[0029]

This measurement result is shown in Table 2

[0030]

[Table 2]

* * * * *	* * * * *
1	0.32
2	1.00
3	0. 12
4	0. 12
5	0.02
6	0.04
7	NT
8	NT
9	NT

[0031]
As for amino acid sequences stated in Sequence Number 3 and 4, from TFE existing at the start of the reaction, it became clear from this result that these sequences possessed decarboxylation activity.

[0032]
Next, chaperone activity measurement of synthetic peptide was done. This measurement method is as follows. 2.98 mM oxalo-acetate (1.7 ml ; 50mM MOPS, 0.1 5M NaCl, pH 7.5) with TFE (0.2 ml) added to a spectroscope cell, at room temperature, with agitation for 5 min, adding 0.1 ml to the measurement sample of 2.0 mM concentration to solution of after stirring,, to obtain a reaction solution total amount as 2 ml, at room temperature, with 1 minute agitation (Iuchi, HS-3B, rotation speed 4). After that, churning was stopped, and absorbance at 285 nm was measured with a spectroscope (Ultroaspec 3100pro, Amersham Bioscienescorp.). Amino acid sequence (0.2 mM) stated in 2.0 mM Sequence Number 3 (or 4) was used as a control sample. Absorbance was reduced after starting the reaction for Sequence Number 3, after 2500 seconds. Similarly, absorbance was reduced for oxalo-acetate using Sequence Number 3.

[0033]
Next, a sample of 0.2 ml test peptide (Sequence Number 5, 6 ,7 or 8) was added in place of TFE of the above-mentioned operation, and agitated for 5 min at room temperature. Then , adding 0.1 ml of sample (Sequence Number 3) of 2.0 mM concentration to solution of after stirring ,to obtain a reaction solution total amount of 2 ml, at room temperature, with 1 minute of agitation (Iuchi, HS-3B, rotation speed 4). After stopping churning, absorbance at 285 nm was measured with the spectroscope. Relative ratio activity for amino acid sequence which is stated in Sequence Number 3 (or 4) of test peptide with below-mentioned Mathematical Formula 2 was sought.

[0034]
[Mathematical Formula 2]

$$[\text{--------}] = (f-e) / (d-e)$$

[0035]
this measurement result is shown in Table 3 .

[0036]

[Table 3]

試料	相対比活性
配列番号3 (200μM) + TFE	1.00
配列番号4 (200μM) + TFE	1.00
配列番号3 (200μM) + 配列番号5 (100μM)	0.12
配列番号3 (200μM) + 配列番号5 (200μM)	0.38
配列番号3 (200μM) + 配列番号6 (100μM)	0.42
配列番号3 (200μM) + 配列番号6 (200μM)	0.44
配列番号3 (200μM) + 配列番号7 (100μM)	NT
配列番号3 (200μM) + 配列番号7 (200μM)	NT
配列番号3 (200μM) + 配列番号8 (100μM)	NT
配列番号3 (200μM) + 配列番号8 (200μM)	NT

[0037]
From this result, as for peptide (Sequence Number 6) of this invention, it became clear to have possessed chaperone activity.

[0038]
Furthermore, chaperone activity measurement of a synthetic peptide was done with another method. This measurement method is as follows. In spectroscope cell, 2 mM Sequence Number 3 (or 4) (0.2 ml), in mixed solution of buffer (50 mM MOPS, 0.1 5M NaCl, pH 7.0) (1.4 ml), was agitated for 48 hours (Iuchi, HS-3B, rotation speed 4) at room temperature including 2 mM measurement sample (Sequence Number 5, 6, 7, 8) (0.2 ml). A total amount of 2.0 ml was obtained after stirring, adding 2.98mM oxalo-acetate (0.2 ml), and then furthermore with 1 minute agitation. Churning was stopped, and absorbance measured at 285 nm at 5000 second after the starting the reaction, using a spectroscope (Ultraspec3100pro, Amersham Biosciences Corp.). As a control sample, absorbance measured with Sequence Number 3 with trifluoro-ethanol (TFE) existing was designated as 1.00.

[0039]
This measurement result is shown in Table 4.

[0040]

[Table 4]

試料	相対比活性
配列番号３ (200μM) + TFE (+)	1.00
配列番号３ (200μM) + TFE (-)	0.59
配列番号４ (200μM) + TFE (+)	0.70
配列番号４ (200μM) + TFE (-)	0.54
配列番号３ (200μM) + 配列番号５ (200μM)	0.62
配列番号３ (200μM) + 配列番号６ (200μM)	0.60
配列番号３ (200μM) + 配列番号７ (200μM)	0.65
配列番号３ (200μM) + 配列番号８ (200μM)	0.57

[0041]
From this result, as for peptide (Sequence Number 6 and 7) of this invention it became clear to have possessed chaperone activity.

[0042]
Next, decarboxylation activity measurement was done using another different method. This measurement method is as follows. In a spectroscope cell, 2 mM Sequence Number 9 (0.2 ml), in mixed solution of buffer (50 mM MOPS, 0.1 5M NaCl, pH 7.0) (1.4 ml), is agitated for 48 hours (Iuchi, HS- 3B,rotation spe< 4) with room temperature including TFE (0.2 ml). An obtained total amount of 2.0 ml including, after stirring, 2.98mM oxalo acetate (0.2 ml), is furthermore agitated for 1 minute. Churning is stopped, and absorbance after 5000 seconds measured with a spectroscope (UltraspecS 100pro, Amersham Biosciences Corp). As control sample, absorbance measured with Sequence Number 2 from trifluoro-ethanol (TFE) existing was designated as 1.00.

[0043]
This measurement result is shown in Table 5 .

[0044]

[Table 5]

*****	****	****
**** 2 200 μM, + TFE (+)	0.195	1.00
**** 2 200 μM + TFE (-)	0.159	
**** 9 200 μM +TFE (+)	0.169	0.61
**** 9 200 μM +TFE (-)	0.147	

[0045]
From this result, as for amino acid sequence which is stated in Sequence Number 9 under TFE existing it became clear to have possessed decarboxylation activity.

[0046]
Next, chaperone activity measurement of chlorpromazine, which is used as antipsychotic drug, was done. This measurement method is as follows. In a spectroscope cell, 2 mM Sequence Number 9 (0.2 ml), in mixed solution of buffer (50 mM MOPS, 0.1 5M NaCl, pH 6.0) (1.4 ml), was agitated for 48 hours (Iuchi,HS- 3B,rotation speed 4) at room temperature, including 2 mM chlopromazine. A total amount of 2.0 ml after stirring , adding 2.98mM oxalo acetate (0.2 ml), was furthermore agitated for 1 minute. Churning was stopped, absorbance measured at 285 nm after 5000 seconds after starting the reaction with a spectroscope (Ultraspec3100pro, Amersham Biosciences Corp.) As control sample, absorbance measured for Sequence Number 9 with chlopromazine existing was designated as 1.00.

[0047]
This measurement result is shown in Table 6 .

[0048]

[Table 6]

試料	相対比活性
ｸﾛﾙﾌﾟﾛﾏｼﾞﾝ (200μM) (pH6.0)	1.00
緩衝溶液中でのｵｷｻﾛｱｾﾃｰﾄの自己分解 (pH6.0)	1.00
配列番号9 (200μM) +ｸﾛﾙﾌﾟﾛﾏｼﾞﾝ (200μM) (pH6.0)	1.31

[0049]
From this result, as for chaperone activity measurement method of this invention it became clear to be able to search low molecular weight organic compound as treatment drug for transmissible spongiform encephalopathy.

[0050]
[Effects of the Invention]
Above-mentioned peptide has molecular chaperone activity, and possesses application for treatment, prevention or diagnostic for transmissible spongiform encephalopathy.

[0051] [Sequence]

<110> BioFrontier Institute Inc. [BioFrontier Kenkyusho KK]
<120> Synthetic Peptide with Chaperone Activity
<130> IY04651
<140>
<141> IP 2002-128976 2002-4-30
IP 2002-200884 2002-7-10
IP 2002-268260 2002-9-13

<160> NO OF <210> SEQ ID NOS : 14

<170> Patent In Ver. 2.1

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<220>
<223> Synthetic Peptide

<220>
<223> Topology is normal chain type
<400> 1
Gly Ile Gly Lys Phe Leu Lys Lys Ala Lys Lys Phe Ala Lys Ala Phe
1 5 10 15
Val Lys lie Leu Lys Lys
20

<210> SEQ ID NO 2
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220>
<223> Synthetic Peptide
<220>
<223> Topology is normal chain type
<400> 2
Leu Ala Lys Leu Leu Lys Ala Leu Ala Lys Leu Leu Lys Lys
1 5 10

<210> SEQ ID NO 3
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220>
<223> Synthetic Peptide
<220>
<223> Topology is normal chain type
<400> 3
Lys Thr Asn Met Lys His Met Ala Gly Ala Ala Ala Ala Gly Ala Val
1 5 10 15
Val Gly Gly Leu Gly
20

<210> SEQ ID NO 4
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220>
<223> Synthetic Peptide
<220>
<223> Topology is normal chain type
<400> 4
Gly Leu Gly Gly Val Val Ala Gly Ala Ala Ala Ala Gly Ala Met His
1 5 10 15
Lys Met Asn Thr Lys
20

<210> SEQ ID NO 5
<211> LENGTH: 13
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
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<223> Synthetic Peptide
<220>
<223> Topology is normal chain type
<400> 5
Asp Ala Pro Ala Ala Pro Ala Gly Pro Ala Val Pro Val
1 5 10

<210> SEQ ID NO 6
<211> LENGTH: 13
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220>
<223> Synthetic Peptide
<220>
<223> Topology is normal chain type
<400> 6
Val Pro Val Ala Pro Gly Ala Pro Ala Ala Pro Ala Asp
1 5 10

<210> SEQ ID NO 7
<211> LENGTH: 13
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220>
<223> Synthetic Peptide
<220>
<223> Topology is normal chain type
<400> 7
Ile Ser Arg Gly Ser Gly Arg Thr Trp Ser Asn Arg Tyr
1 5 10

<210> SEQ ID NO 8
<211> LENGTH: 13
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220>
<223> Synthetic Peptide

<220>
<223> Topology is normal chain type
<400> 8
Ile Ser Asp Gly Ser Gly Asp Thr Trp Ser Asn Asp Tyr
1 5 10

<210> SEQ ID NO 9
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
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<223> Synthetic Peptide
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<223> Topology is normal chain type
<220>
<223> C- terminus is
<400> 9
Lys Thr Asn Met Lys His Met Ala Gly Ala Ala Ala Ala Gly Ala Val
1 5 10 15
Val Gly Gly Leu Gly
20

<210> SEQ ID NO 10
<211> LENGTH: 13
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
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<220> PEPTIDE
<13>
Xaa represents Asp or Glu
<400> 10
Val Pro Val Ala Pro Gly Ala Pro Ala Ala Pro Ala Xaa
1 5 10

<210> SEQ ID NO 11
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<213> ORGANISM: Artificial Sequence
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<223> Synthetic Peptide
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<3>
Xaa represents Asp, Glu or Arg
<220> PEPTIDE
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Xaa represents Asp, Glu or Arg
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PEPTIDE
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Xaa represents Asp, Glu or Arg
<400> 11
Ile Ser Xaa Gly Ser Gly Xaa Thr Trp Ser Asn Xaa Tyr
1 5 10

<210> SEQ ID NO 12
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<223> Synthetic Peptide
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PEPTIDE
<2>
Xaa represents Asp, Glu or Arg
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PEPTIDE
<7>
Xaa represents Asp, Glu or Arg
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PEPTIDE
<11>
Xaa represents Asp, Glu or Arg
<400> 12
Tyr Xaa Asn Ser Trp Thr Xaa Gly Ser Gly Xaa Ser He
1 5 10

<210> SEQ ID NO 13
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<212> TYPE: PRT
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<220>
<223> Synthetic Peptide
<400> 13
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1 5 10 15
Val Gly Gly Leu Gly
20

<210> SEQ ID NO 14
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<213> ORGANISM: Artificial Sequence
<220>
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<400> 14
Gly Leu Gly Gly Val Val Ala Gly Ala Ala Ala Ala Gly Ala Met His
1 5 10 15
Lys Met Asn Thr Lys
20

Drawings

Molecular determinants of the physicochemical properties of a critical prion protein region comprising residues 106–126

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Prion diseases are marked by the cerebral accumulation of conformationally modified forms of the cellular prion protein (PrP^C), known as PrP^{res}. The region comprising the residues 106–126 of human PrP seems to have a key role in this conformational conversion, because a synthetic peptide homologous with this sequence (PrP106–126) adopts different secondary structures in different environments. To investigate the molecular determinants of the physicochemical characteristics of PrP106–126, we synthesized a series of analogues including PrP106–126 H_D, PrP106–126 A and PrP106–126 K, with L-His → D-His, His → Ala and His → Lys substitutions respectively at position 111, PrP106–126 NH_2 with amidation of the C-terminus, PrP106–126 V with an Ala → Val substition at position 117, and PrP106–126 VNH_2 with an Ala → Val substitution at position 117 and amidation of the C-terminus. The analysis of the secondary structure and aggregation properties of PrP106–126 and its analogues showed the following. (1) His^{111} is central to the conformational changes of PrP peptides. (2) Amidation of the C-terminal Gly^{126} yields a predominantly random coil structure, abolishes the molecular polymorphism and decreases the propensity of PrP106–126 to generate amyloid fibrils. (3) PrP106–126 V, carrying an Ala → Val substitution at position 117, does not demonstrate a fibrillogenic ability superior to that of PrP106–126. However, the presence of Val at position 117 increases the aggregation properties of the amidated peptide. (4) Amyloid fibrils are not required for neurotoxicity because the effects of PrP106–126 NH_2 on primary neuronal cultures were similar to those of the wild-type sequence. Conversely, astroglial proliferation is related to the presence of amyloid fibrils, suggesting that astrogliosis in prion encephalopathies without amyloid deposits is a mediated effect rather than a direct effect of disease-specific PrP isoforms.

Key words: amyloid, prion protein peptides, secondary structure modifications.

INTRODUCTION

Prion diseases such as scrapie of sheep and goats, bovine spongiform encephalopathy, and Creuzfeldt–Jakob disease ('CJD') and Gerstmann–Sträussler–Scheinker disease (GSS) of humans are characterized by the accumulation of abnormal forms of the cellular prion protein (PrP^C), termed PrP^{res}, in the brain [1]. In contrast with PrP^C, PrP^{res} is partly resistant to digestion with protease and has a marked tendency to form insoluble aggregates and amyloid fibrils [2–4]. The accumulation of PrP^{res} and PrP amyloid in the brain is thought to be responsible for the nerve cell degeneration, astrogliosis and activation of microglial cells observed in prion-related encephalopathies [5–7].

NMR studies of recombinant murine PrP indicate that the normal protein is composed of two structurally distinct moieties: an extended N-terminal segment (residues 23–125) with features of a flexibly disordered polypeptide chain, and a well-defined globular domain (residues 126–231) with three α-helices and a two-stranded anti-parallel β-sheet [8–10]. The transition from PrP^C to PrP^{res} involves a striking conformational change with a decrease in α-helical secondary structure (from 42% to 30%) and a remarkable increase in β-sheet content (from 3% to 43%) [11,12]. This rearrangement is accompanied by the acquisition of abnormal physicochemical properties, including insolubility in non-denaturing detergents and partial resistance to digestion with proteinase K.

Previous studies have shown that a synthetic peptide homologous with residues 106–126 of human PrP (PrP106–126) exhibits some of the pathogenic and physicochemical properties of PrP^{res} [5–7,13–15]. Like PrP^{res}, this peptide causes nerve cell death by apoptosis and induces hypertrophy and proliferation of astrocytes and activation of microglial cells *in vitro* [5–7]. It is noteworthy that the neurotoxicity of the peptide requires the expression of endogenous PrP, which is consistent with the observation that neuronal death in scrapie infection *in vivo* is dependent on PrP^C synthesis [16,17]. It also increases the membrane microviscosity of a variety of cells, including neurons and astrocytes [18,19]. PrP106–126 shows a remarkable conformational polymorphism, acquiring different secondary structures in different environments [15]; nevertheless, it tends to adopt a β-sheet conformation in buffered solutions and aggregates into amyloid fibrils that are partly resistant to digestion with protease. These data indicate that the PrP region including residues 106–126 might be the nido at which conformational change is initiated in the conversion of PrP^C to PrP^{res}. This view is supported by the observation that the N-terminal half of PrP

Abbreviations used: GSS, Gerstmann–Sträussler–Scheinker; PrP, prion protein; PrP^C, cellular PrP; PrP^{res}, pathological and protease-resistant isoform of PrP; PrP106–126, synthetic peptide comprising residues 106–126 of human PrP; PrP106–126 H_D, with L-His → D-His substitution at position 111; PrP106–126 A, with His → Ala substitution at position 111; PrP106–126 K, with His → Lys substitution at position 111; PrP106–126 V, with Ala → Val substitution at position 117; PrP106–126 NH_2, with amidation at the C-terminus; PrP106–126 VNH_2, with Ala → Val substitution at position 117 and amidation of the C-terminus; TFE, 2,2,2-trifluoroethanol.

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(residues 23–125) is highly flexible and more susceptible to structural transitions than the C-terminal globular domain, and that the deletion of residues 23–88 does not prevent the conversion of PrP^{C} to PrP^{res}, whereas the ablation of residues 108–121 or 122–140 together with residues 23–88 does so [20].

The present study was undertaken to investigate the molecular determinants of the conformational polymorphism of PrP106–126 and its propensity to form amyloid fibrils. Several peptide analogues containing amino acid changes were synthesized and their physicochemical properties were compared with the those of wild-type sequence. The selection of amino acid changes was based on the assumption that the conformational polymorphism and fibrillogenic properties of PrP106–126 depend at least in part on the presence of an ionizable residue (i.e. His^{111}) between the hydrophilic and hydrophobic domains of the peptide, and on electrostatic and hydrophobic interactions between molecules conferring stability to the β-sheet configuration. We also analysed the effects of the Ala → Val substitution at position 117, a mutation linked to GSS disease. This effect was evaluated on the wild-type protein and the C-terminal amidated form of PrP106–126 (PrP106–126 NH_2). The latter was used because the amidation of the C-terminus greatly decreases the fibrillogenic properties of the peptide and allows the more effective detection of the influence of the amino acid substitution. Finally, we investigated how the physicochemical changes resulting from the amidation of the C-terminus and the Ala → Val substitution at residue 117 affected the biological activity of the peptide.

EXPERIMENTAL

Peptide synthesis

The following peptides were used for the study: PrP106–126 (K^{106}TNMKHMAGAAAAGAVVGGLG; single-letter codes); PrP106–126 H_D, with an L-His → D-His substitution at position 111; PrP106–126 A (K^{106}TNMKAMAGAAAAGAVVGGLG), with an His → Ala substitution at position 111; PrP106–126 K (K^{106}TNMKKMAGAAAAGAVVGGLG), with a His → Lys substitution at position 111; PrP106–126 V (K^{106}TNMKHMAGAAVAGAVVGGLG), with an Ala → Val substitution at position 117; PrP106–126 NH_2 (K^{106}TNMKHMAGAAAAGAVVGGLG-NH_2), with amidation at the C-terminus; and PrP106–126 VNH_2 (K^{106}TNMKHMAGAAVAGAVVGGLG-NH_2), with an Ala → Val substitution at position 117 and amidation at the C-terminus. The peptides were synthesized by solid-phase chemistry and purified by reverse-phase HPLC, as described previously [15]. Purity was always greater than 95%.

Preparation of peptide solutions

Peptides were dissolved in deionized water at 0.75 or 1.5 mg/ml (stock solutions). Under these conditions they were soluble, as deduced by the absence of a visible pellet after centrifugation at 13000 *g* for 10 min. The stock solutions were stable for 2 weeks at −80 °C, as determined by reverse-phase HPLC (see below). Aliquots of stock solutions were added to 200 mM phosphate buffer, pH 5.0 or 7.0, to obtain final peptide concentrations of 0.25, 0.50, 0.75 or 1.0 mg/ml phosphate buffer. The actual concentration of each peptide in phosphate buffer was determined by HPLC analysis immediately after preparation of the samples and was used as the zero-time value in sedimentation experiments. To investigate how the α-helix-stabilizing solvent 2,2,2-trifluoroethanol (TFE) influenced the secondary structure, the peptides were first suspended in phosphate buffer, pH 5.0, at a concentration of 0.5 mg/ml; TFE was then added to the samples to a final concentration of 50% (v/v).

CD spectroscopy

The analysis was performed on 0.25 mg/ml peptide suspensions in phosphate buffer, pH 5.0 and 7.0, or phosphate buffer containing TFE, prepared as described above. The spectra were recorded after incubation for 1 h in quartz cells, with an optical path of 0.1 cm, by using a Jasco J-500 dichograph (Tokyo, Japan) at a scan speed of 2 nm/min. Mean residue ellipticities were calculated by using the following equation: $(\theta)_M = 3300AM/Cl$, where A is the observed dichroic absorbance, l is the path length in cm, C is the concentration of the peptide in g/l and M is the mean residue weight. The percentages of the secondary structure of the peptides were calculated by the method of Yang et al. [21].

Turbidity measurements

The analysis was performed on 0.25 mg/ml peptide suspensions in phosphate buffer, pH 5.0 and 7.0. The turbidity of the samples was determined immediately after their preparation (zero time) and after incubation for 24 h at room temperature, with a Perkin–Elmer Lambda 10 spectrophotometer at 600 nm.

Sedimentation experiments

The study was performed on 0.75 mg/ml peptide suspensions in phosphate buffer, pH 5.0. Aliquots of 20 μl were incubated at 37 °C for 0, 1, 2, 4 and 24 h, then chilled on ice and centrifuged at 13000 *g* for 10 min at 4 °C. Supernatant (2 μl) was injected into an HPLC apparatus equipped with a 3.9 mm × 150 mm Delta-Pak C_{18} column, a model P 4000 pump, a UV 2000 variable-wavelength detector, an AS 3000 autosampler (Waters, Milford, MA, U.S.A.) and an SP 4400 integrator operated in both peak height and area modes (Thermo Separations Products, Riviera Beach, FL, U.S.A.). MilliQ water/acetonitrile (75:25, v/v) containing 0.1% (v/v) trifluoroacetic acid was used as eluent, at a flow rate of 1 ml/min. The column eluates were monitored at 214 nm. The peptide concentrations in the supernatant at different times were expressed as percentages of the corresponding values determined at zero time.

Light and electron microscopy

Peptides were suspended in phosphate buffer, pH 5.0, at a final concentration of 1 mg/ml. After incubation for 24 h at 20 °C, 50 μl of each suspension was air-dried on gelatin-coated slides, stained with 1% (w/v) aqueous thioflavin S and analysed by fluorescence microscopy. For electron microscopy, 5 μl of suspension was applied to Formvar-coated nickel grids, negatively stained with 5% (w/v) uranyl acetate and observed in an electron microscope (EM 109, Zeiss, Germany) at 80 kV.

Nerve cell cultures

Cerebral cortex was dissected from fetal rat brains on embryonic day 17. Cortical cells were dissociated and plated in 24-well dishes (3.5×10^5 cells per dish) at 37 °C in humidified air/CO_2 (19:1), as described previously [5]; 24 h after being plated, the cultures were exposed to PrP106–126, PrP106–126 NH_2 or

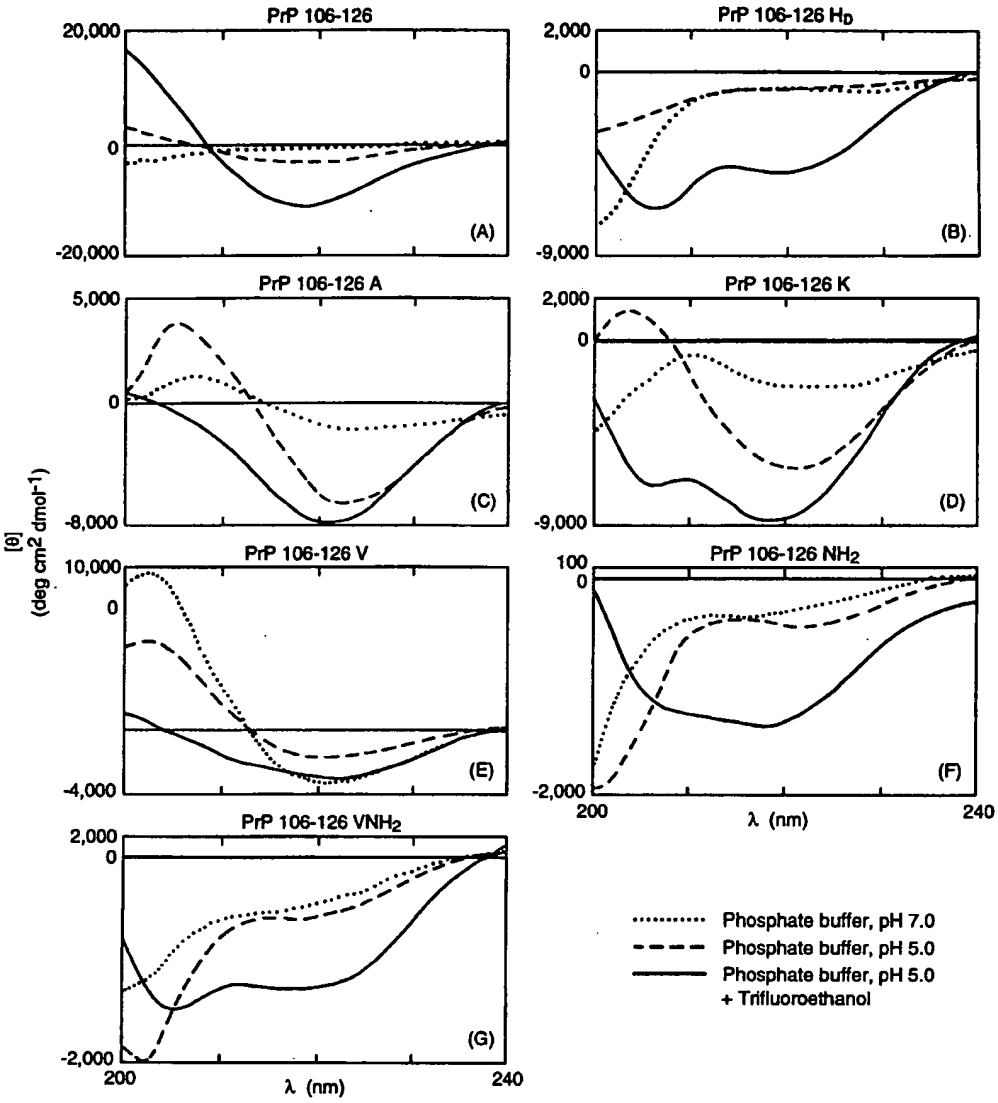

Figure 1 CD spectra of PrP106–126 and its analogues

PrP106–126 VNH_2 at 50 μM for 7 days. Control cultures were exposed to vehicle only. Cell viability was then assessed by staining with Crystal Violet [0.5% in water/methanol (4:1)]. After washing, cells were dried and the staining intensity was determined densitometrically with an image analyser (IBAS 2.0; Zeiss, Cologne, Germany) [22].

Astroglial cultures

Glial cell cultures were prepared from newborn rat pups as described [6]. After 10 days of culture, flasks containing mixed glial cells were given fresh medium and shaken at 37 °C for 12–16 h. The supernatant containing microglia and oligodendrocytes was removed and replaced with fresh Dulbecco's modified Eagle's medium. The adherent cells (astrocytes) were then exposed for 5 min to 0.25% trypsin. After the addition of an equal volume of Dulbecco's modified Eagle's medium/10% (v/v) fetal calf serum, the cell suspension was centrifuged and the pellet was resuspended in medium containing 10% fetal calf serum. Cells were plated at a density of 5×10^4 cells/ml [20] and exposed to PrP106–126, PrP106–126 NH_2 or PrP106–126 VNH_2 at 50 μM for 7 days. Control cultures were exposed to vehicle only. Glial proliferation was assessed by staining with Crystal Violet and densitometric analysis [23,24].

RESULTS

Secondary structure of PrP peptides as deduced by CD spectroscopy

In a previous study we showed that PrP106–126 can adopt different conformations in different environments, e.g. a random coil structure in deionized water, a combination of random coil and β-sheet in 200 mM phosphate buffer, pH 7.0, a β-sheet conformation in phosphate buffer, pH 5.0, and an α-helical structure in TFE. A remarkable finding was the stability of the β-sheet secondary structure, which was not affected by the addition of TFE to a solution of the peptide in phosphate buffer, pH 5.0 [15].

Table 1 Percentages of secondary structure in peptide PrP106-126 and its analogues

Results are means ± S.E.M. for at least three different experiments.

Row	Peptide	pH	TFE	Secondary structure (%) α-Helix	β-Sheet	β-Turn	Random coil
A	PrP106–126	5.0	–	22±3	61±4	–	17±4
B		7.0	–	17±2	40±5	4±1	39±2
C		5.0	+	28±5	58±3	2±1	12±5
D	PrP106–126 H_D	5.0	–	5±1	12±5	5±3	78±7
E		7.0	–	4±2	4±3	11±5	81±9
F		5.0	+	48±6	16±3	5±2	31±7
G	PrP106–126 A	5.0	–	15±4	53±6	14±2	18±5
H		7.0	–	13±3	51±5	16±2	20±5
I		5.0	+	25±8	50±10	9±1	16±5
J	PrP106–126 K	5.0	–	22±3	51±6	12±3	15±2
K		7.0	–	9±2	24±5	18±2	51±7
L		5.0	+	40±8	42±10	2±1	16±5
M	PrP106–126 V	5.0	–	25±3	58±8	2±1	20±2
N		7.0	–	14±2	44±5	4±2	38±1
O		5.0	+	22±5	59±9	1±0	18±2
P	PrP106–126 NH_2	5.0	–	5±2	11±5	11±7	73±13
Q		7.0	–	4±3	8±4	12±4	76±11
R		5.0	+	51±7	25±5	5±2	19±7
S	PrP106–126 VNH_2	5.0	–	5±2	15±2	9±5	71±10
T		7.0	–	5±1	19±4	9±2	67±7
U		5.0	+	42±8	22±3	6±3	30±6

To unravel the molecular determinants of these structural properties, we generated PrP106–126 analogues with modifications of single amino acids (substitution of D-His, Ala or Lys for L-His[111]; substitution of Val for Ala[117]; amidation of the C-terminus with or without the substitution of Val for Ala[117]) and analysed the effects of these changes on the conformational polymorphism of the peptides and the stability of the β-sheet conformation. Figure 1 and Table 1 show the CD spectra and percentages of secondary structure of PrP106–126 analogues in phosphate buffer, pH 5.0 and 7.0, and in phosphate buffer, pH 5.0, after the addition of TFE.

The characteristics of PrP106–126 were substantially altered by modifications of His[111]. PrP106–126 H_D showed a striking decrease in β-sheet and α-helical content, with a parallel increase in random coil structure; this effect was observed at both pH 5.0 and 7.0 (Figure 1B, and Table 1, rows D and E). The addition of TFE to PrP106–126 H_D solutions in phosphate buffer markedly enhanced the α-helical content (Figure 1B, and Table 1, row F). PrP106–126 A showed a high proportion of β-sheet secondary structure at both pH 5.0 and 7.0, as indicated by a positive peak with a maximum at 206 nm and a broad negative band centred on 220 nm (Figure 1C, and Table 1, rows G and H); the only difference between the spectra recorded at neutral and acidic pH was the signal intensity, which was much stronger at pH 5.0. The addition of TFE to PrP106–126 A in phosphate buffer resulted in a slight increase in α-helix (Figure 1C, and Table 1, row I). PrP106–126 K adopted a predominantly β-sheet conformation in phosphate buffer at pH 5.0 (Figure 1D, and Table 1, row J); the β-sheet content decreased at pH 7.0 and the random coil became the prevalent secondary structure (Figure 1D, and Table 1, row K). The addition of TFE to PrP106–126 K in phosphate buffer caused an ordered arrangement with equal contributions of α-helix, as deduced by two negative bands at 206 and 220 nm, and β-sheet, as indicated by a more intense, broad negative absorption at 220 nm (Figure 1D, and Table 1, row L).

Table 2 Turbidity measurements on PrP106-126 and its analogues

Peptides were suspended in 200 mM phosphate buffer, pH 5.0 or 7.0, at a concentration of 0.25 mg/ml; and the difference in D_{600} between zero time and after incubation for 24 h at room temperature was calculated. Results are means ± S.E.M. for at least four determinations. *$P < 0.05$ compared with the peptide suspension at pH 5.0 (Student's t test).

Peptide	ΔD_{600} between 0 and 24 h of incubation pH 5.0	pH 7.0
PrP106–126	0.085±0.008	0.114±0.032*
PrP106–126 H_D	0.071±0.010	0.078±0.014
PrP106–126 A	0.121±0.005	0.126±0.002
PrP106–126 K	0.075±0.010	0.080±0.011
PrP106–126 V	0.081±0.001	0.110±0.001*
PrP106–126 NH_2	0.064±0.003	0.066±0.003
PrP106–126 VNH_2	0.069±0.002	0.096±0.011*

The substitution of Val for Ala[117] (PrP106–126 V) had no significant effect on the spectral features and structural stability of PrP106–126, which had a high β-sheet content in phosphate buffer (Figure 1E, and Table 1, rows M and N) and also in the presence of TFE (Figure 1E, and Table 1, row O). Amidation of the C-terminal Gly[126] (PrP106–126 NH_2) decreased the propensity to adopt the β-sheet structure in phosphate buffer, at both pH 5.0 and 7.0; under these conditions, the peptide showed a random coil arrangement, as indicated by the strong negative band at 200 nm (Figure 1F, and Table 1, rows P and Q). The addition of TFE to PrP106–126 NH_2 in phosphate buffer, pH 5.0, caused a striking increase in α-helical structure (Figure 1F, and Table 1, row R). The substitution of Val for Ala[117] of the amidated peptide (PrP106–126 VNH_2) slightly changed the spectral features in phosphate buffer, at both pH 5.0 and 7.0; this change consisted of an increased intensity of the negative band at 220 nm, indicating a higher degree of β-sheet secondary structure (Figure 1G, and Table 1, rows S and T). The addition of TFE to PrP106–126 VNH_2 in phosphate buffer, pH 5.0, resulted in an increased α-helical content (Figure 1G, and Table 1, row U); this increase was less pronounced than that observed for PrP106–126 NH_2.

Aggregation properties of PrP106–126 peptides

The ability of PrP106–126 analogues to form macroaggregates was first assessed by turbidity measurements. The peptides were suspended in phosphate buffer, pH 5.0 or 7.0, at 0.25 mg/ml, and the difference in absorbance between zero time and after incubation for 24 h at room temperature was calculated (Table 2). At zero time, no significant differences in optical density were observed between the different peptide suspensions at both pH 5.0 and 7.0; in a typical experiment at pH 5.0, the following initial values (means ± S.E.M. for four replicates) were recorded: PrP106–126, 0.058±0.005; PrP106–126 H_D, 0.055±0.004; PrP106–126 A, 0.065±0.008; PrP106–126 K, 0.055±0.09; PrP106–126 V, 0.062±0.004; PrP106–126 NH_2, 0.051±0.008; PrP106–126 VNH_2, 0.053±0.009. After incubation for 24 h, PrP106–126 suspensions showed an increase in turbidity values that was higher at pH 7.0 than at pH 5.0. Similar figures were obtained with PrP106–126 V and PrP106–126 VNH_2, sug-

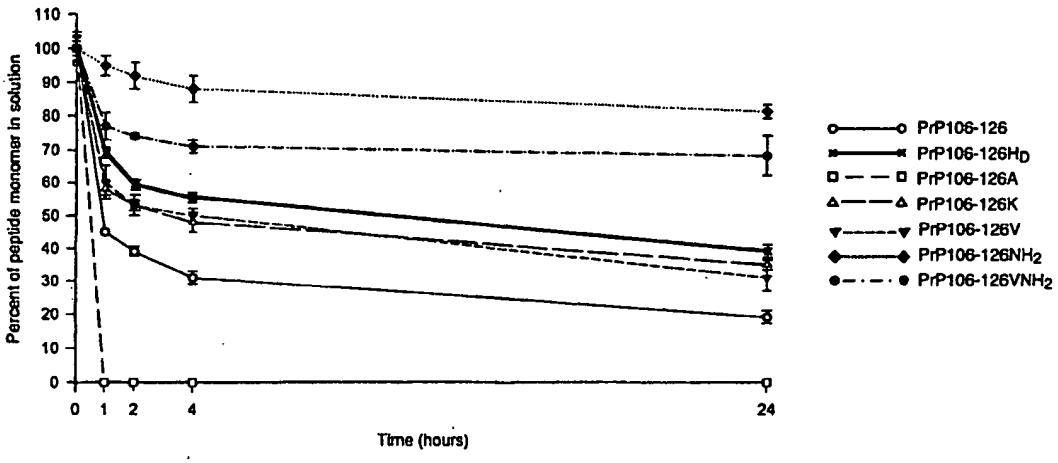

Figure 2 Sedimentation pattern of PrP106–126 and its analogues

Each point represents the percentage of peptide monomer in the 13000 *g* supernatant fraction of peptide solutions in 200 mM phosphate buffer, pH 5.0, incubated at room temperature for different durations. Results are means ± S.E.M. for three determinations.

gesting that the neutral pH and the amino acid change linked to GSS disease enhance these peptides' ability to aggregate. In contrast, the increases in turbidity of the analogues carrying the substitution of D-His, Ala or Lys for L-His[111] or the amidation of Gly[126] were similar at pH 5.0 and 7.0; however, the magnitude of the increase differed between these peptides, being highest in PrP106–126 A and lowest in PrP106–126 NH_2. With regard to PrP106–126 A, the increase in turbidity occurred very rapidly and reached a plateau within 1 h (results not shown), suggesting that this peptide is highly insoluble in buffered solutions. It is noteworthy that the decrease in aggregation capacity and the sensitivity to pH variations of PrP106–126 NH_2 were partly restored by the substitution of Val for Ala[117].

These results were consistent with those obtained by HPLC determination of the proportion of the peptides that was not precipitated by centrifugation. Whereas turbidimetry revealed the aggregated fraction, sedimentation enabled us to quantify the soluble fraction. This was lowest for PrP106–126 A, followed by PrP106–126, PrP106–126 V, PrP106–126 K, PrP106–126 H_D, PrP106–126 VNH_2 and PrP106–126 NH_2. All peptides showed a decrease in soluble fraction with time; the decrease was extremely rapid for PrP106–126 A, which was already undetectable in the supernatant after 1 h owing to its high insolubility (Figure 2).

Ultrastructural and staining properties of PrP peptide assemblies

The nature of the aggregates generated by PrP106–126 and its analogues was determined by electron microscopy after negative staining and by fluorescence microscopy after treatment with thioflavin S. As reported previously, PrP106–126 formed dense meshes of straight, unbranched fibrils 4–8 nm in diameter and up to 2.0 μm long (Figure 3A). The fibrillar assemblies showed yellow fluorescence after staining with thioflavin S. Similar findings were observed with peptides with the substitution of Lys for His[111] (results not shown) and of Val for Ala[117] (Figure 3D). Conversely, PrP106–126 H_D showed a striking decrease in density, length and diameter of the fibrils (Figure 3B).

The most remarkable change in the fibrillogenic properties of the peptide was seen with PrP106–126 A, of which the aggregates consisted primarily of amorphous material and, to a smaller

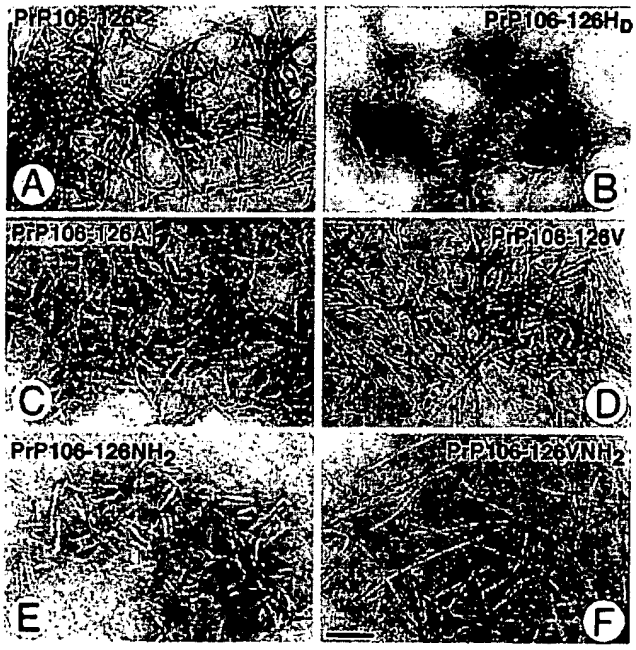

Figure 3 Electron micrographs of fibrils generated *in vitro* by PrP106–126 and its analogues

Shown are PrP106–126 (**A**), PrP106–126 H_D (**B**), PrP106–126 A (**C**), PrP106–126 V (**D**), PrP106–126 NH_2 (**E**) and PrP106–126 VNH_2 (**F**). Scale bar, 100 nm.

extent, of thin, short filamentous structures with an irregular profile (Figure 3C), lacking the staining properties of amyloid. PrP106–126 NH_2 also had a lower fibrillogenic ability than PrP106–126; this was partly restored in PrP106–126 VNH_2, whereas PrP106–126 NH_2 generated a few straight fibrils 4–8 nm in diameter and less than 0.2 μm in length (Figure 3E). The morphology and staining properties of the peptide assemblies formed by PrP106–126 VNH_2 were similar to those of PrP106–

Table 3 Effects of PrP106-126 analogues on nerve and astroglial cells

Cultures of neurons and astrocytes were exposed for 7 days to the peptides [5,6]. Neuronal viability and astroglial proliferation were estimated by a colorimetric method. Results are expressed as percentage of control and are means ± S.E.M. for six to twelve determinations. *$P < 0.01$ compared with the relevant control group (Dunnett's test).

	Nerve cell viability		Astroglial proliferation	
Peptide	Control	Peptide	Control	Peptide
PrP106–126	100 ± 5.7	23.6 ± 5.8*	100 ± 3.0	265 ± 10*
PrP106–126 NH_2	100 ± 6.2	23.0 ± 7.9*	100 ± 6.0	119 ± 4.0
PrP106–126 VNH_2	100 ± 4.8	39.9 ± 9.1*	100 ± 10	164 ± 2.0*

126 (Figure 3F); however, the fibril density was significantly lower than for the native sequence.

Biological activities of PrP106–126 analogues with different aggregation properties

Previous studies showed that the highly fibrillogenic PrP106–126 is neurotoxic and induces astroglial proliferation *in vitro*. To investigate whether these biological properties are related to the aggregation state of the peptide, primary cultures of neurons and astrocytes were exposed to analogues with diverse abilities to assemble into amyloid-like fibrils. In particular, PrP106–126 was compared with the poorly fibrillogenic PrP106–126 NH_2 and with PrP106–126 VNH_2, which had an intermediate propensity to aggregate. The study showed that the neurotoxicity of PrP106–126 analogues was not dependent on their fibrillogenic properties because all peptides induced a similar decrease in nerve cell viability after 7 days of treatment at 50 μM. Conversely, the effects on astrocytes seemed to be related to the aggregation state because PrP106–126 NH_2 was unable to induce astroglial proliferation, whereas PrP106–126 VNH_2 showed partial activity (Table 3).

DISCUSSION

The sequence comprising residues 106–126 of human PrP corresponds to a highly conserved region of the molecule, located in the flexibly disordered N-terminal segment adjacent to the structurally well-defined globular domain [9,10]. This region is required for the conversion of PrP^C into PrP^{res} [20]. Studies *in vitro* suggest that it might have a central role in the conformational change of PrP and in the pathogenesis of tissue changes associated with the accumulation of PrP^{res} in the brain [5–7,12–17].

A recent study [25] on recombinant PrP corresponding to human residues 91–231 showed that reduction of the disulphide bond between Cys^{179} and Cys^{214} resulted in a conformational change from a predominantly α-helical to a β-sheet structure. Most of this rearrangement seemed to occur within the C-terminal domain, whereas residues 91–126 were found to be unstructured in both the α-helical and β-sheet forms of the protein. The discrepancy between this observation and our results could be due to profound differences in structure and behaviour when the PrP region comprising residues 106–126 is isolated as a peptide or is a part of the PrP polypeptide chain. However, previous studies showed that the disulphide bond is required for PrP^{res} formation [20]; thus the change from α-helical to β-sheet structure after reduction of the protein might be different from that occurring *in vivo*. In this regard, it is noteworthy that the protease-resistant core of PrP^{res} corresponds approximately to residues 90–231 and includes the segment 90–125. Because this segment is flexible and protease-sensitive in PrP^C, it must undergo a significant conformational change in the conversion to PrP^{res} that precludes its accessibility to proteases.

We have previously shown that the synthetic peptide PrP106–126 adopts different conformations in different environments, although it has a high propensity to form stable β-sheet structures and to assemble into amyloid fibrils [13–15]. Because the secondary structure of the peptide is markedly influenced by pH, its physicochemical properties might be at least partly related to His^{111}, which is the only residue that changes in ionization state between pH 5.0 and 7.0 and is located in a critical region between the N-terminal polar head (KTNMKH) and the long hydrophobic tail (MAGAAAAGAVVGGLG). The relevance of His^{111} to the conformational change of PrP and the pathogenesis of prion diseases is suggested by the observations that (1) human PrP^C undergoes proteolytic cleavage between Lys^{110} and His^{111}, whereas the abnormal PrP species associated with prion diseases do not [26], and (2) transgenic mice expressing Syrian hamster PrP carrying the double substitution of the more hydrophobic residue Ile for Lys^{110} and His^{111} spontaneously develop spongiform changes and astrogliosis of the grey matter, similar to those observed in scrapie-infected mice [27].

The present study further supports the view that His^{111} is a critical site of the protein. The substitution of D-His^{111} for L-His^{111} abolished the pH-dependent conformational polymorphism of PrP106–126, caused a loss of stability of the β-sheet structure and decreased the peptide's fibrillogenic ability. Conceivably these effects are related to the fact that the orientation of the imidazole moiety of D-His is opposite to that of L-His; this might cause steric hindrance with neighbouring Lys^{110} and Met^{112} residues, inducing the formation of a random coil structure. In contrast, the substitution of the highly hydrophobic, non-ionizable residue Ala for His^{111} caused the peptide to become insoluble in buffered solutions. Although PrP106–126 A showed a high proportion of β-sheet secondary structure, it formed mainly amorphous aggregates. This is most probably due to the prevalence of the intramolecular hydrophobic forces of the lipophilic residues on the kinetics of fibril assembly. Unexpectedly, replacing His^{111} with Lys (a polar residue that is always ionized in the pH range 5.0–7.0) modified the pH-dependent structural polymorphism of the peptide, although it did not affect its ability to form amyloid fibrils. As mentioned above, His^{111} is located between the hydrophobic and hydrophilic portions of PrP106–126; this molecular environment might affect the pK_a of Lys at this site. Furthermore, the β-sheet secondary structure of PrP106–126 K was largely maintained in TFE, suggesting that its stability depends on an ionized residue at position 111.

Our results support previous observations that the β-sheet content is a key factor for amyloid fibril formation. The only exception was the substitution of Ala for His^{111}, as discussed above. Conversely, there was no simple and direct relation between the amount of β-sheet secondary structure of PrP106–126 analogues and their aggregation properties, as deduced by CD spectroscopy and turbidimetry respectively. The aggregation abilities of PrP106–126 and PrP106–126 V were greater at pH 7.0 than pH 5.0, despite both peptides' having a higher content of β-sheet structure at acidic pH. Furthermore, the quantity of aggregates of PrP106–126 K was similar at pH 5.0 and 7.0, although the β-sheet content was distinctly larger at pH 5.0. These apparent inconsistencies are due to the fact that CD spectroscopy and turbidimetry explore peptide populations in

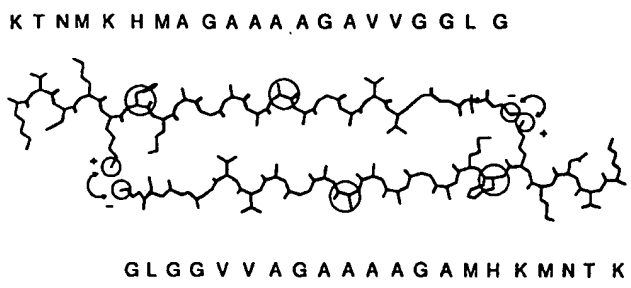

Figure 4 Proposed model of β-sheet formation by PrP106–126, showing an antiparallel alignment of two molecules of PrP106–126

The two ends of the secondary β-sheet structure are stabilized by ion-pair interactions. The residues His^{111}, Val^{117}, the N-terminal Lys^{106} and C-terminal Gly^{126} are highlighted.

different physical states (the former detects the soluble fraction, whereas the latter reveals insoluble macroaggregates), so a correlation between the two might not always be apparent. PrP106–126 and PrP106–126 V are more soluble at pH 5.0 than at pH 7.0 owing to the protonation of His^{111}, which decreases the propensity of the peptides to aggregate by antagonizing the intramolecular hydrophobic forces of the lipophilic core. This allows the presence of a larger population of monomers or oligomers in solution that are capable of adopting a β-sheet secondary structure. With PrP106–126 K, His^{111} was replaced by a residue that is not sensitive to pH change; aggregation was therefore not affected.

The contribution of the C-terminal carboxy group to the physicochemical properties of amyloid peptides has been investigated by Terzi et al. [28,29], by using the fragment 25–35 of the β-protein involved in the pathogenesis of Alzheimer disease. Removal of the C-terminal electric charge by amidation led to a predominantly random-coil structure and inhibited fibril formation. On the basis of this observation, we amidated the C-terminal Gly^{126} of PrP106–126, seeking to decrease the fibrillogenic ability of the peptide. This in turn allowed us to assess the relationship between aggregation state and biological activity as well as the influence of the substitution Val for Ala^{117} on the physicochemical characteristics. The amidation of the C-terminus abolished the molecular polymorphism of PrP106–126 yielding a predominantly random coil structure at both neutral and acidic pH, and increased the sensitivity to the helix-promoting solvent TFE. Interestingly, although this change also decreased the propensity of the peptide to generate amyloid fibrils, the fibrillogenic ability was not completely abolished. A possible interpretation of these results is that PrP106–126 molecules assemble in an anti-parallel β-pleated sheet structure, which is stabilized by head-to-tail ion-pair interactions between Lys^{106} and Lys^{110} and the carboxy group of Gly^{126} (Figure 4). Elimination of the charge at the C-terminus shifts the equilibrium towards the random-coil monomeric state. However, the finding that PrP106–126 NH_2 still forms a limited number of fibrils suggests that van der Waals interactions involving the hydrophobic segment of the peptide contribute to fibril assembly.

Among prion-related encephalopathies, GSS disease is characterized by the highest degree of parenchymal amyloid [30]. This disorder segregates with mutations of the PrP gene. Because one of these mutations (i.e. $Ala^{117} \rightarrow$ Val) is contained in the PrP106–126 region, we studied its effects on the peptide's conformational and fibrillogenic properties. Valine is a well-known β-sheet structure promoter [31,32] that increases the hydrophobic profile of the C-terminal part of PrP106–126. By molecular dynamic simulations of a similar peptide, PrP109–122, Kazmirski et al. [33] found that substitution of Val for Ala^{117} was the only helix-destabilizing modification of a variety of replacements with hydrophobic amino acid residues [33]. PrP106–126 V did not demonstrate a fibrillogenic ability superior to that of PrP106–126. However, the importance of this substitution was apparent by comparing PrP106–126 NH_2 and PrP106–126 VNH_2, because neither had a carboxy contribution to aggregation. The presence of Val at position 117 increased the aggregation properties of the amidated peptide; fibril morphology was very similar to that of PrP106–126.

With regard to the relationship between aggregation state and biological activity of PrP peptides, our results indicate that amyloid fibrils are not required for neurotoxicity because the effects of PrP106–126 NH_2 on primary neuronal cultures were similar to those of the wild-type sequence. Conversely, under our experimental conditions, astroglial proliferation seemed to be related to the presence of amyloid fibrils. Therefore it is possible that astrogliosis in prion encephalopathies without amyloid deposits is a mediated effect (e.g. microglia-dependent) rather than a direct effect of disease-specific PrP isoforms.

In conclusion, our results support the view that the PrP region spanning residues 106–126 is susceptible to conformational transition and might be the nido at which the conversion of PrP^C to PrP^{res} is initiated. Furthermore, evidence suggests that, within this region, His^{111} is important to the structural flexibility as well as the stability of the newly formed β-sheet structure. PrP106–126 and PrP106–126 NH_2 can be considered to be complementary models for investigation of the pathogenesis of prion diseases. The former is suitable for short-term studies *in vitro*; however, its use is limited by its poor manageability. This is at least partly related to the free carboxy ion on Gly^{126}, which is moreover not present in the intact protein. Removal of this charge results in a peptide that retains neurotoxic activity and mimics the slow kinetics of fibril formation observed *in vivo* in prion-related encephalopathies.

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